KISSPEPTIN AND GONADOTROPIN-RELEASING HORMONE SIGNALING IN SKELETAL MUSCLE

A Thesis

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Master of Science

by

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ABSTRACT

Chronic inflammation contributes to skeletal muscle atrophy and impaired regenerative capacity. Previous research has identified skeletal muscle inflammatory susceptibility (MuIS), or the ability to manage and respond to inflammation, as a predictor of failed skeletal muscle regeneration and regrowth following surgery. This inflammatory susceptibility is associated with human aging and likely contributes to the adverse structural, metabolic, and functional tissue remodeling that occurs in aged adults. The etiology for heightened inflammation in skeletal muscle is unclear, particularly in healthy young adults. Research that elucidates the mechanisms by which inflammation impairs skeletal muscle regeneration is necessary for identifying potential therapeutic targets.

Previous research from our laboratory identified kisspeptin-1 (KISS1) as a highly differentially expressed gene (DEgene) associated with lower inflammatory susceptibility (MuIS-) in human skeletal muscle. When the MuIS- group was compared to a group with an improved hypertrophic response to resistance training, gonadotropin-releasing hormone (GnRH) signaling emerged as a top canonical pathway. In the present study, we aimed to investigate the potential relationship between KISS1/GnRH signaling and inflammation in skeletal muscle and elucidate the relevant signaling pathways.

The presence of the GnRH receptor (GnRHr) was assessed in the skeletal muscle of mice genetically modified to constitutively express β-galactosidase at the GnRHr promoter (RG) and wild type (WT) mice using a β-galactosidase gene detection assay. Cell culture experiments using differentiated human muscle progenitor cells (*h*MPCs) and immortalized mouse skeletal muscle stem cells (C2C12 cells) were performed to determine activation of canonical GnRH signaling (i.e., mitogen-activated protein kinases [MAPKs]), following treatment with Buserelin, a GnRH analog. The National Center for Biotechnology Information (NCBI) Gene Expression

Omnibus (GEO) datasets were searched for pathological conditions in which KISS1, KISS1 receptor (KISS1r), GnRH, or GnRHr was differentially expressed in skeletal muscle and under inflammatory conditions in other organs. Results were analyzed using the NCBI software GEO2R, which contains R packages from the Bioconductor project. Lastly, the potential relationship between GnRH and inflammatory signaling was assessed using reverse transcription-polymerase chain reaction (RT-PCR) analysis of interleukin 6 (IL-6) following treatment with Buserelin and the inflammatory cytokine tumor necrosis factor alpha (TNFα).

GnRHr was detected at the protein level in RG but not WT muscle, which validates the presence of GnRHr in skeletal muscle tissue. GnRH signaling may activate extracellular signal-related kinase (ERK) in human skeletal muscle. Additionally, the transcription factor cAMP response element-binding protein (CREB) and Jun N-terminal kinase (JNK) have the capability to respond to Buserelin and Antide treatments in differentiated C2C12 cells. KISS1/GnRH expression was found to be downregulated in several inflammatory myopathies, including juvenile dermomyositis, Duchenne muscular dystrophy, amyotrophic lateral sclerosis, facioscapulohumeral dystrophy, tibial muscular dystrophy, and myotonic dystrophy type 2. GnRH did not impact the acute TNFα-mediated increase in IL-6 mRNA levels in skeletal muscle.

Our data confirm that GnRHr protein is present and functional in skeletal muscle. Further, our results demonstrate that KISS1/GnRH have a relationship with inflammation that is impacted with inflammatory susceptibility and myopathies with signaling that may occur through the MAPK signaling pathway. Future research should focus on identifying phenotypes associated with a lack of KISS1 or GnRH signaling in skeletal muscle; KISS1/GnRH is a potential therapeutic target to attenuate heightened inflammation in disease states.

BIOGRAPHICAL SKETCH

Lauren Varvatos, BS, is a graduate student in the MS program in the Division of Nutritional Sciences at Cornell University and is expecting to graduate in August 2019. Lauren is enrolled in the individualized track, studying molecular and human nutrition with a minor concentration in physiology. Prior to enrolling in the MS program, she received a BS in Nutritional Sciences from Michigan State University in East Lansing, MI in May 2017. During her undergraduate studies, she gained experience in community nutrition research and global public health.

Lauren's current research interests center on potential therapeutic targets to reduce chronic inflammation in skeletal muscle. During her graduate studies, she presented research projects at two conferences: The Advances in Skeletal Muscle Biology in Health and Disease Conference and the American Society for Nutrition Conference. For one year, Lauren mentored an undergraduate student in the lab, including training the student in lab techniques and experimental design. She worked in the Human Metabolic Research Unit (HMRU) in the clinical lab. In the HMRU, Lauren conducted research participant visits, assisted with muscle biopsies, processed tissue, and trained undergraduate students in blood processing and data entry. Lauren served as a graduate teaching assistant for two courses: Human Anatomy and Physiology Laboratory for two semesters and Methods in Nutritional Sciences for one semester, where she assisted with teaching dissections and laboratory procedures, wrote exam questions, created assignments, mentored students, and graded assignments. Lauren designed and taught two nutrition and physiology mini courses in a local elementary school and middle school as a part of the Graduate Student Outreach Program. She co-designed and conducted two Expanding Your Horizons workshops at Cornell.

Lauren will matriculate into the 2019 entering class at Wayne State University, School of Medicine in Detroit, MI in July. As a physician, Lauren would like to incorporate current research in molecular, human, and community nutrition into the practice of endocrinology. Her specific career interests include transgender medicine, type 2 diabetes, and obesity, especially in underserved populations. Lauren plans to become a physician committed to wellness, compassion, and health equity.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP-activated protein kinase	AMPK
Analysis of variance	ANOVA
Anteroventral periventricular nucleus	AVPV
Beta-galactosidase	β-galactosidase
Calcium/calmodulin-dependent protein kinases	CaMK
cAMP response element-binding protein	CREB
Cyclic adenosine monophosphate	cAMP
Differentially expressed genes	DEgenes
Differentiation media	DM
Dulbecco's Modified Eagle Media	DMEM
Early growth response 1	EGR1
Extracellular acidification rate	ECAR
Extracellular signal-regulated kinases	ERK1/2
F-box only protein 32	FBX032
Fibroblast growth factor	FGF
Fluorescence activated cell sorting	FACS
Follicle-stimulating hormone	FSH
Gonadotropin-releasing hormone	GnRH
Gonadotropin-releasing hormone receptor	GnRHr
Growth media	GM
G protein-coupled receptor 54	GPR54
Human muscle progenitor cells	*h*MPCs
In vitro fertilization	IVF
Ingenuity Pathway Analysis	IPA
Inositol-(1,4,5)-trisphosphate	IP3
Insulin-like growth factor 1	IGF-1
Interleukin 1 beta	IL-1β
Interleukin 6	IL-6
Jun N-terminal kinase	JNK
Kisspeptin 1	KISS1
Kisspeptin 1 receptor	KISS1r
Kisspeptin/neurokinin B/dynorphin	KNDy
Lipopolysaccharide	LPS
Luteinizing hormone	LH
Matrix metalloprotease 9	MMP9
Mammalian target of rapamycin complex 1	mTORC1
Mitogen-activated protein kinase	MAPK
Muscle inflammatory susceptible positive	MuIS+
Muscle inflammatory susceptibility negative	MuIS-
Muscle progenitor cell	MPC
Myogenic differentiation 1	MYOD1
Myogenin	MYOG
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Ovarian hyperstimulation syndrome	OHSS
Oxidative phosphorylation	OXPHOS

Oxygen consumption rate	OCR
Phosphate buffered saline	PBS
Phospholipase C	PLC
Polycystic ovarian syndrome	PCOS
Reverse-transcription polymerase chain reaction	RT-PCR
Principal component analysis	PCA
Principal component 1	PC1
Principal component 2	PC2
Protein kinase A	PKA
Protein kinase C	PKC
Rosa26/GnRHr internal ribosomal entry site Cre	Rosa Gric
RNA sequencing	RNA-seq
Satellite cells	SC
Transforming growth factor beta	TGFβ
Tripartitie motif containing 63	TRIM63
Tumor necrosis factor alpha	TNFα
Wild type	WT

CHAPTER 1. INTRODUCTION

1.1 Skeletal Muscle Structure and Function

Skeletal muscle is the largest organ system in the human body, in individuals with a normal body mass index (BMI) (1). Muscle tissue is structurally comprised of multinucleated cells (myofibers), which are encapsulated by a cell membrane (the sarcolemma) and further arranged in bundles (fascicles). Fascicles, surrounded by a connective tissue layer (the perimysium), are bundled together to form entire muscles, which are covered by the epimysium layer. The myofiber of the muscle contains that contractile unit, the sarcomere, which is important for the physical functions of muscle. Skeletal muscle is a remarkably dynamic and resilient tissue with functions ranging from voluntary movement and nutrient homeostasis to temperature regulation and protection (2). Given the central functions of the muscle, maintenance of skeletal muscle structural and functional homeostasis is vital to overall human health and quality of life.

1.2 Skeletal Myogenesis

Throughout the life-course the skeletal muscle undergoes damage that requires tissue repair, regeneration, and even regrowth. Skeletal muscle damage may be caused by eccentric muscle contractions; surgery; and crush, burn, or blunt injuries, among others (3). When tissue damage occurs, a process known as regeneration takes place, allowing the tissue to regain functionality. The regenerative capacity of skeletal muscle relies on the resident adult stem cells, known as satellite cells (SCs), that surround myofibers in the space between the sarcolemma and basal lamina (4). In an essential step of the carefully orchestrated myogenic program, quiescent SCs are activated in response to injury-associated cues. Activation of the SCs can trigger symmetric or asymmetric proliferation, which yields a muscle progenitor cell (myoblast) and a

SC. Myoblasts further differentiate (myocytes) and fuse to form or repair the multinucleated myofibers (myotubes in culture). Less severe damage causes SCs to fuse with and strengthen existing myofibers, while more severe damage promotes the development of new myofibers (3, 5). With asymmetric cell division, a portion of the activated SCs return to a quiescent state and maintain the reserve of stem cells. Given the role of SCs in the generation of myofibers, SCs, harvested from skeletal muscle tissue that are further cultured, are an optimal model to study not only myogenesis, but also factors that impact skeletal muscle health.

1.3 Inflammation in Skeletal Muscle

The acute inflammatory response following skeletal muscle injury, is essential for normal recovery and regeneration of the muscle (6). Inflammatory signals are required to activate SCs. In response to muscle damage, the proinflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin 1β (IL-1β) are released by the muscle itself or by resident macrophages (7,8). These proinflammatory cytokines activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. NF-κB is a transcription factor that upregulates the expression of additional proinflammatory genes, notably TNFα, IL-1β, and interleukin 6 (IL-6) (Figure 1). These proinflammatory cytokines are important in recruiting neutrophils and additional macrophages that promote proteolysis and clear tissue debris following injury (9). Macrophages also secrete growth factors that are important for SC and myoblast proliferation. TNFα also activates SC proliferation, in addition to accelerating the transition from G1 to S phase of the cell cycle and participating in the regulation of myogenesis. (10, 11, 12). Within the last several decades, it has become apparent that precisely regulated crosstalk between the inflammatory response and muscle cells is necessary for successful myogenesis.

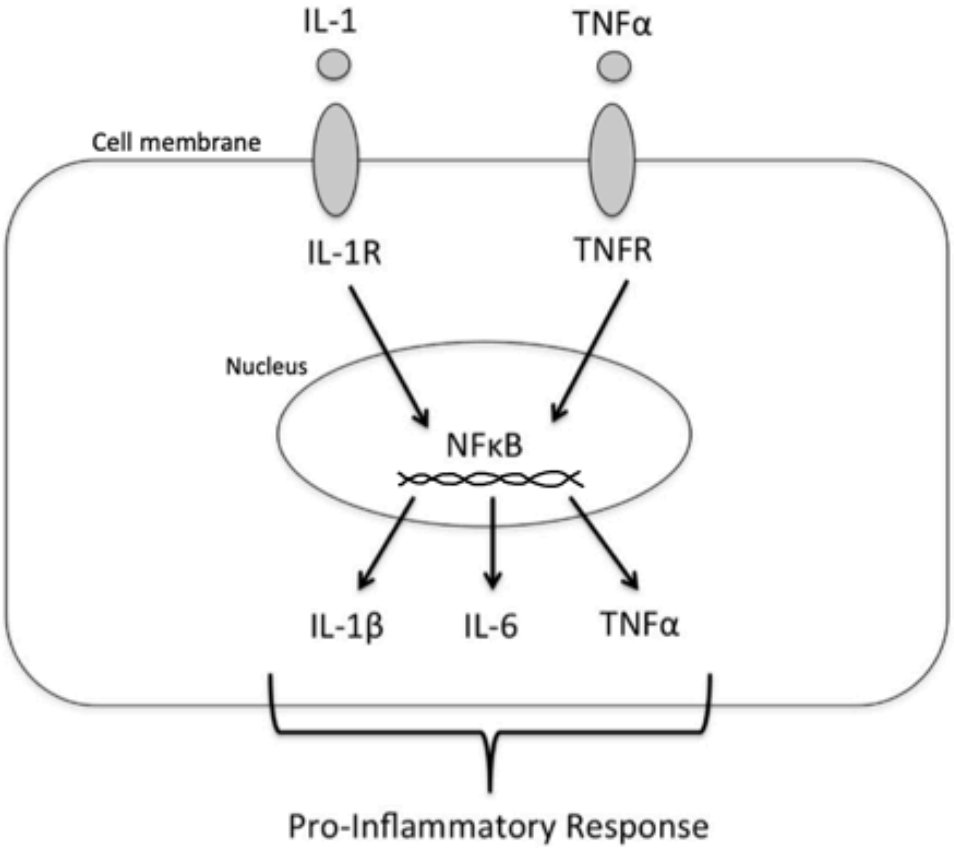


Figure 1. Inflammatory Cytokine Signaling.

Despite the necessity of acute inflammatory signaling in the early stages of muscle regeneration and repair, chronically heightened inflammation contributes to skeletal muscle atrophy and impaired regenerative capacity (13). Previous research identified skeletal muscle inflammatory susceptibility, or the ability to manage and respond to inflammation, as a predictor of failed skeletal muscle regeneration and regrowth following surgery (14, 15). This inflammatory susceptibility is associated with human aging and likely contributes to the adverse structural, metabolic, and functional tissue remodeling that occurs in aged adults (14). Proinflammatory cytokines play central roles in muscle wasting and tissue remodeling. In the skeletal muscle of cachectic tumor-bearing mice, circulating levels of IL-6 were associated with

suppression of protein synthesis by inhibiting the activation of the mammalian target of rapamycin complex 1 (mTORC1), a complex that is important for protein synthesis and cell proliferation (16). Similarly, infusion of TNFα or IL-1β into skeletal muscle *in vivo* inhibited protein synthesis and promoted catabolism in rats (17). TNFα also prevented differentiation of myoblasts *in vitro* by repressing MyoD, an early marker of myogenic commitment (18, 19).

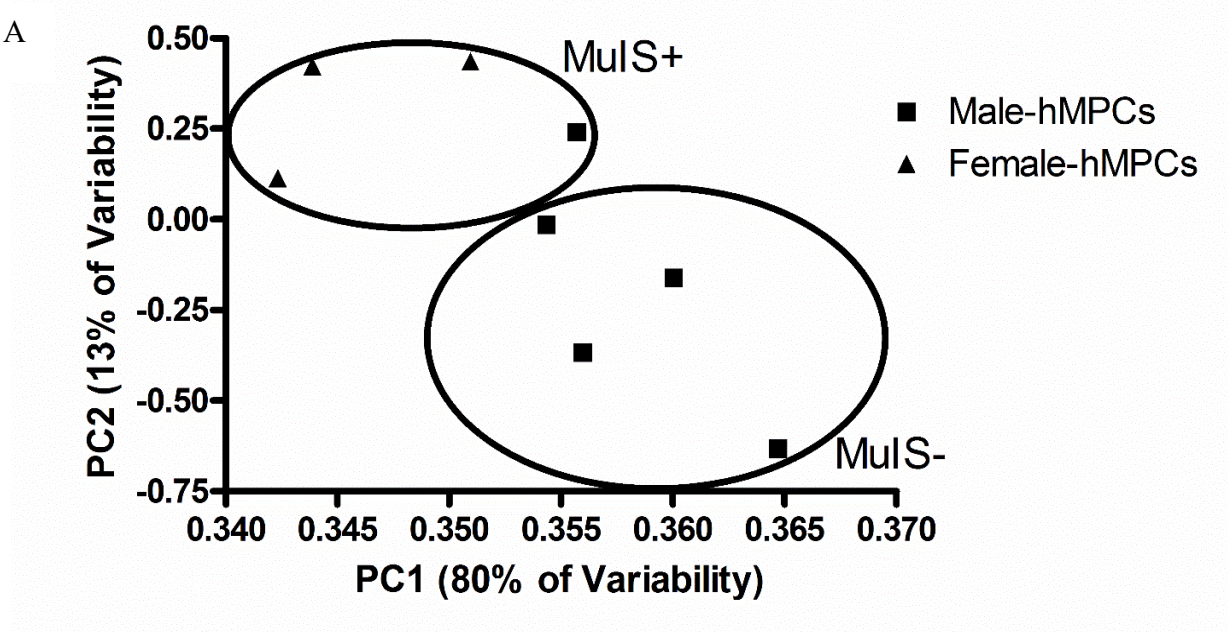
Chronic inflammation-induced skeletal muscle deterioration is governed by similar proinflammatory signaling pathways that regulate the acute inflammatory response to injury. Upregulation of the NF-κB proinflammatory pathway has been implicated in animal models of cancer cachexia (20, 21, 22). Activation of NF-κB positively regulates the expression of murine ring finger-1 (MuRF1), an E3 ubiquitin ligase protein that is central in muscle atrophy and wasting (23). The p65 subunit of NF-κB, which potentiates canonical NF-κB signaling, has been associated with impaired myogenesis. In a mouse model with reduced p65 NF-κB activity, SC proliferation, myogenic differentiation, and regenerative capacity of skeletal muscle were enhanced *in vitro* and *in vivo* (24).

Myopathies, diseases of the skeletal muscle, are often closely associated with dysregulated inflammatory signaling (25). Idiopathic inflammatory myopathies are characterized by persistent infiltration of innate immune cells, overactive protein degradation, and pathological tissue remodeling leading to fibrosis (26). In the context of aging, myopathy, or other conditions associated with a heightened inflammatory state, chronic inflammation in skeletal muscle is a hallmark of impaired myogenesis and muscle deterioration. While dysregulation of inflammatory signaling pathways are known to underlie muscle protein degradation, disruption of the myogenic program, and impediments in successful tissue regeneration, therapies to attenuate chronic inflammatory signaling in muscle are lacking.

1.4 KISS1/GnRH—Novel Mechanism Associated with Skeletal Muscle Inflammation

In previous research from our laboratory (27), cultured and differentiated human primary SCs, from young male and female donors, were separated into two clusters based on the expression of pro-inflammatory cytokines IL-6, IL-1β, and TNFα expression; the two clusters were myocytes with (MuIS+) and without (MuIS-) inflammatory susceptibility. RNA-sequencing (RNA-seq), on a subset of MuIS+ (n = 4) and MuIS- (n = 4), was used to confirm the donor myocyte culture classification with principal components analysis (PCA) (Figure 2A) and to determine the mechanisms underlying inflammation susceptibility in the MuIS+. We identified 573 differentially expressed (DEgenes) (178 downregulated and 395 upregulated) between MuIS+ and MuIS-. The RNA-seq data was validated using quantitative, real-time polymerase chain reaction (RT-PCR) for five genes that were differentially expressed between MuIS+ and MuIS- clusters (Figure 2B). Genes used for validation included pro-inflammatory, degradative, and myogenic genes. Further, Ingenuity Pathways Analysis (IPA) was performed for pathway enrichment to identify potential mechanisms underlying inflammatory susceptibility.

A



B

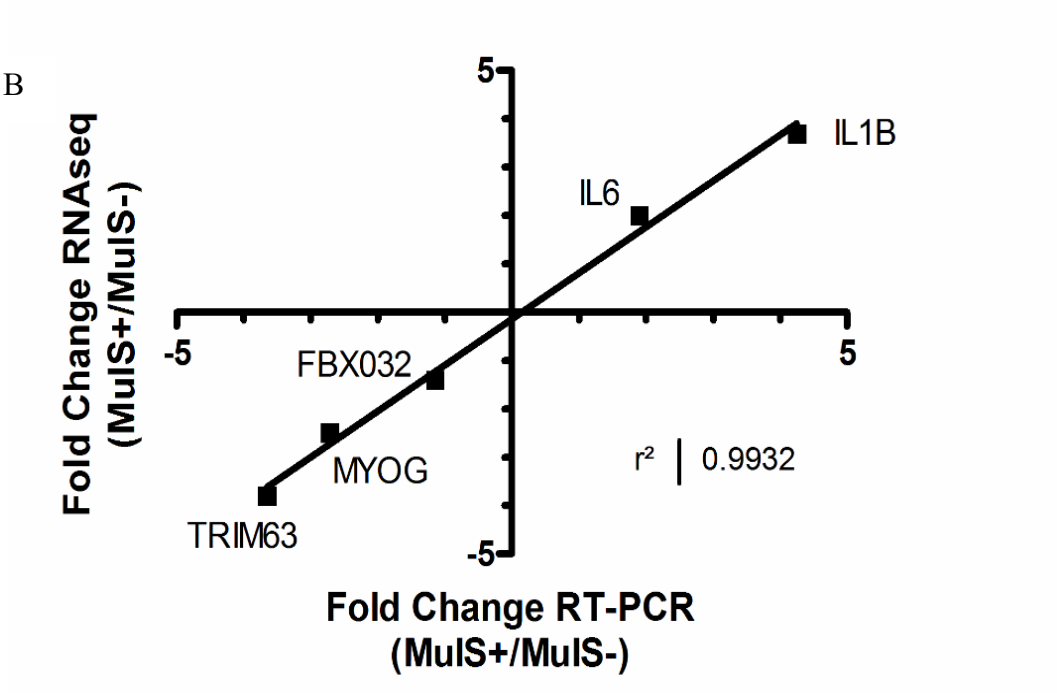


Figure 2. MuIS+ and MuIS- Clusters. (A) Principal component analysis of the log_2FPKM values was used to reduce the dimensionality and unbiasedly cluster the RNA-seq data. (B) Validation plot comparing the fold-change between RT-PCR and RNA-seq for five genes that were differentially expressed between MuIS+ and MuIS- clusters.

Unexpectedly, we identified that kisspeptin-1 (KISS1) had the greatest difference in gene expression between MuIS+ and MuIS- (downregulated 21.4-fold [MuI+/MuIS-]) Further, KISS1 was identified as a node in the top network (Network 1) of DEgenes following pathway analysis. Network 1 had over-representation of DEgenes related to *Organ Morphology, Reproductive System Development and Function, and Tissue Morphology* (Figure 3).

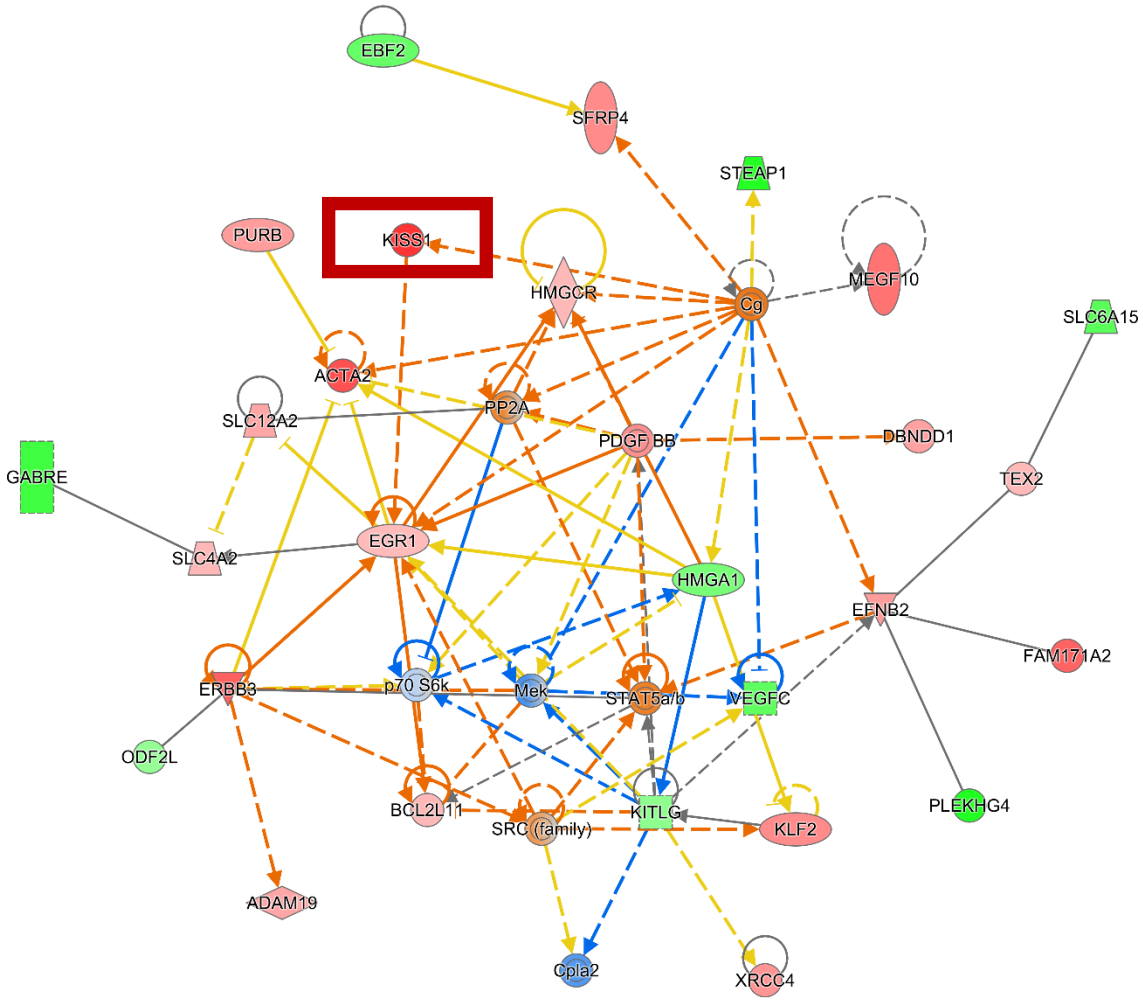


Figure 3. Network of DEgenes. KISS1 was identified as a node in a top network of DEgenes following IPA. Nodes represent a gene or gene product and edges represent signals, such as activation or repression, that go from one node to another. Red, upregulated; Green, downregulated.

Further, we compared the canonical pathways identified using the DEgenes for MuIS+/MuIS- with the canonical pathways of DEgenes from non- versus extreme hypertrophic responders. Non- and extreme-hypertrophic responder classification was determined via myofiber growth following 16 weeks of progressive resistance training (28). Non-responders had over-representation DEgenes in pro-inflammatory functional categories (29). Intriguingly, gonadotropin-releasing hormone (GnRH) signaling was identified as a top canonical pathway,

down-regulated, in MuIS+ myocytes and non-responder skeletal muscle tissue. KISS1 has regulatory roles through GnRH, but the relationship among KISS1, GnRH, and inflammation in the skeletal muscle is unknown.

1.5 Canonical Roles of KISS1 and GnRH

Kisspeptins are a family of proteins encoded by the KISS1 gene in humans. Kisspeptins are cleaved from a 145 amino acid precursor peptide and include the fragments kisspeptin-54, kisspeptin-10, kisspeptin-13, and kisspeptin-14. All kisspeptins bind to the kisspeptin receptor (KISS1r), also known as G protein-coupled receptor 54 (GPR54), with equal affinity (30). KISS1r is coupled to the Gαq/11 signaling pathway, where activation of the primary effector phospholipase C (PLC) results in the production of inositol-(1,4,5)-trisphosphate (IP3) and diacylglycerol, mobilization of calcium, and activation of protein kinase C (PKC) and extracellular signal-regulated kinases 1 and 2 (ERK1/2). KISS1/KISS1r signals through these secondary messengers in multiple cell types (31).

The mitogen-activated protein kinase (MAPK) signaling pathways regulate cellular processes including cell proliferation, differentiation, and survival or apoptosis (Figure 4) (32, 33). MAPK pathways, including ERK1/2, p38 MAPK, and c-Jun N-terminal kinase (JNK), are key regulators of the inflammatory response in various human diseases (34). ERK, a known regulator of cell proliferation, is regulated by KISS1 in many tissues (31). ERK is involved in the response to DNA damage and is dysregulated in many human cancers, leading to tumorigenesis and resistance to anticancer therapies (35). KISS1 binding to its receptor can activate ERK1/2 through both the Gαq/11 and β-arrestin-2 pathways (36).

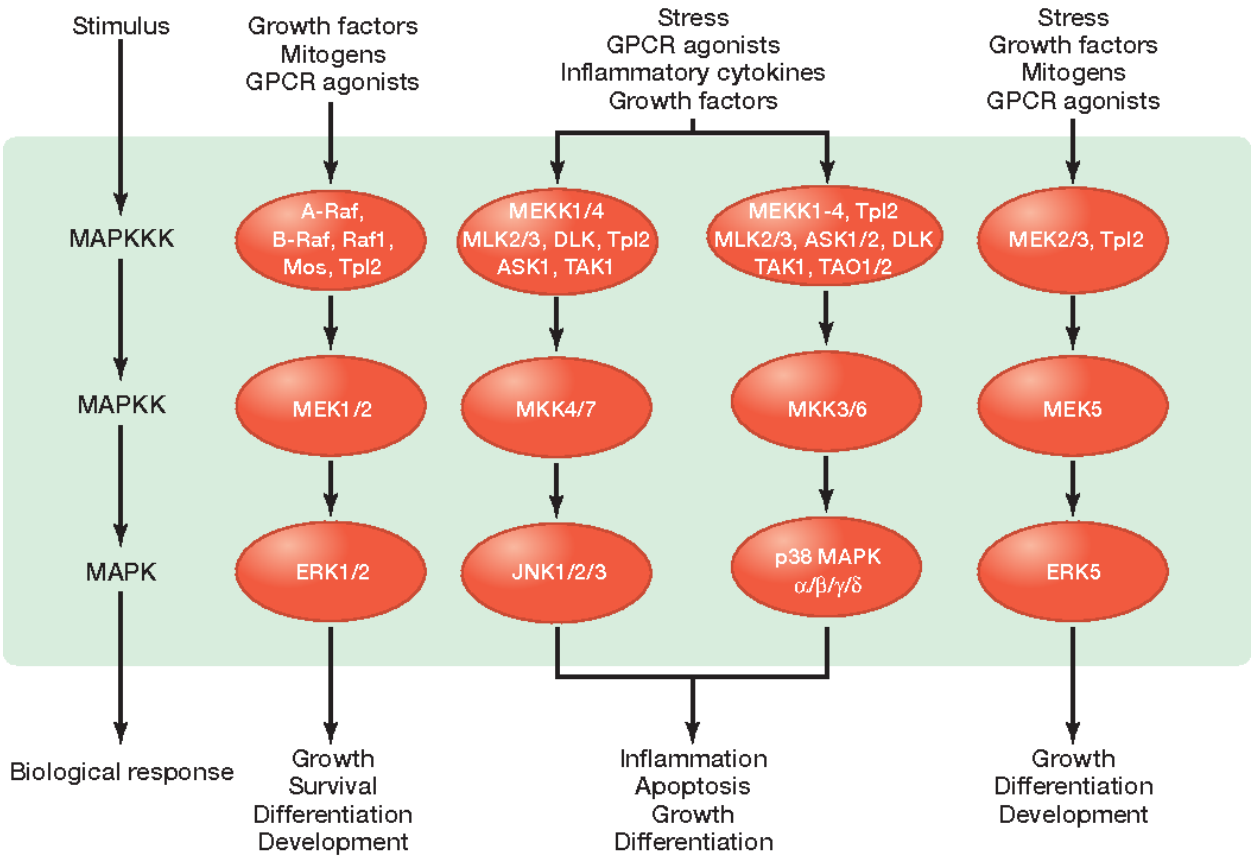


Figure 4. The MAPK Pathways. The ERK and JNK pathways are key regulators of the inflammatory response and are regulated by KISS1 and/or GnRH in many tissues. Taken from Morrison, 2012

KISS1 is widely known for its regulatory role in the hypothalamic-pituitary-gonadal axis, which occurs through the activation of GnRH neurons. KISS1 and KISS1r are expressed by neurons in the arcuate nucleus and anteroventral periventricular nucleus (AVPV) of the hypothalamus (38, 30). In response to KISS1, the hypothalamic decapeptide hormone GnRH is released by GnRH neurons into the hypophyseal portal capillary system and acts on gonadotropes of the anterior pituitary gland to stimulate the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH travel through systemic circulation to promote the synthesis and release reproductive hormones from the gonads, leading to ovarian

follicular development and testicular spermatogenesis (39). KISS1/KISS1r signaling is required for the onset of puberty and normal reproductive function in mammals (30, 37, 39).

KISS1 has also been proposed as a major regulator of placentation and fetal development based on its roles in inhibition of trophoblast migration and invasion (40). Because of its ability to induce oocyte maturation, KISS1 has been effectively used as fertility treatment in women undergoing in vitro fertilization (IVF), conferring a lower risk of ovarian hyperstimulation syndrome (OHSS) than human chorionic gonadotropin (hCG) treatment (41). This method of IVF is particularly relevant for treating infertility in women with polycystic ovarian syndrome (PCOS), who are at a five-fold higher risk of developing OHSS (41).

1.6 KISS1 and GnRH Signaling

The ERK signaling cascade is also responsible for the major biological effects of GnRH. LH transcription is dependent on ERK-induced expression of EGR1, and ERK1/2 ablation in the pituitary of female mice results in anovulation (42). In an immortalized human cervical cancer cell line (HeLa cells) transduced to express the GnRH receptor (GnRHr), treatment with GnRH induced a maximum response of ERK1/2 activation at 5 min followed by a rapid decrease to nearly basal levels by 60 min (43). In the pituitary gonadotrope, GnRH activates ERK1/2 in a calcium-dependent manner, facilitating voltage-gated L-type channels to open and allow calcium influx (44). In systems such as the pituitary, GnRHr couples to the Gs alpha subunit (Gαs) and stimulates cAMP (45). GnRHr-induced calcium-dependent cAMP elevation has been proposed as a Gαs-independent mechanism of GnRH signaling (44, 46, 47). GnRH also signals transiently through JNK MAPK in rat pituitary gonadotropes. While GnRH-induced activation of ERK requires extracellular calcium, GnRH signaling through JNK relies on intracellular calcium (48). In addition to ERK and JNK, GnRH signals through cAMP response element-binding protein

(CREB), a member of the leucine zipper superfamily of transcription factors. CREB is involved in the stress response and regulates cell survival and proliferation in most human tissues (49).

GnRH signaling is precisely controlled by several factors upstream of KISS1. Estrogen, a primary sex hormone, exerts both negative and positive feedback on GnRH secretion by acting on kisspeptin/neurokinin B/dynorphin (KNDy) neurons in the arcuate nucleus and kisspeptin-releasing AVPV nucleus, respectively (37, 49). The positive feedback response to estrogen in AVPV neurons causes an LH surge only in females, and this sexual dimorphism allows for sex-specific hormonal responses (49). In addition to its role in the reproductive system, estrogen stimulates skeletal muscle repair and regeneration via SC activation and proliferation and has been associated with a suppressed inflammatory response in skeletal muscle in response to injury (50, 51, 52, 53).

1.7 KISS1/GnRH in Cellular Processes

KISS1 is potent suppressor of metastasis and has been shown to inhibit the TNFα-mediated activation of NF-κB signaling in breast cancer cells (54). Metastasis suppressors, defined by their ability to inhibit metastasis without blocking orthotopic tumor growth, are central therapeutic targets in human cancers (55). Following transfection with KISS1 and its constitutive expression, metastasis was suppressed in human malignant melanoma cells and in a highly metastatic sub-clone (56). KISS1 is downregulated during human melanoma progression *in vivo*, likely due to a loss of heterozygosity and inactivation of a tumor suppressor gene localized on the same chromosome (57, 58). KISS1 suppresses the migration of pancreatic cancer cells and simultaneously activates ERK1/2 (59, 60). In gastric cancer, reduced KISS1 gene expression is associated with distant metastases, worsened survival, and lymph node and liver metastases tumors (61, 62, 55). Since their discovery, kisspeptins have been explored as

therapeutic targets for melanoma, thyroid cancer, bladder cancer, esophageal carcinoma, gastric cancer, hepatocellular carcinoma, and breast cancer (30). Originally, cytoskeleton remodeling was proposed as a mechanism of KISS1/KISS1r action in breast cancer cells, as KISS1-transfectants spread on type-IV collagen more quickly than control cells (56). As KISS1 acts through MAPK pathways, regulation of cell proliferation and motility have also been implicated in the anti-metastatic capabilities of KISS1 (30).

1.8 KISS1/GnRH Signaling and Inflammation

KISS1 negatively regulates inflammatory signaling. In cervical and ovarian cancer cell lines, KISS1/KISS1r binding inhibited chemokine signaling (63). KISS1 has been shown to inhibit the TNFα-mediated activation of the NF-κB pathway in breast cancer cells and dephosphorylate NF-κB in fibrosarcoma cells, resulting in dissociation from the matrix metalloprotease 9 MMP-9 (MMP-9) promoter via a MAPK-independent mechanism (54, 64). Further, a knockdown of a metastasis-promoting gene in breast cancer cells led to the upregulation of KISS1, inhibition of MMP-9, lower levels of NF-κB p65/50 subunits in the nucleus, and blunted metastatic response to treatment with TNFα (65).

While KISS1 has been shown to negatively regulate inflammatory pathways, several studies have also identified that KISS1/KISS1r signaling is dysregulated with inflammation. An acute inflammatory challenge with lipopolysaccharide (LPS) has been shown to decrease KISS1 mRNA expression in the hypothalamus (66) and microarray analysis of rat trigeminal ganglion tissue after treatment with complete Freund's adjuvant, which stimulates an acute inflammatory response, demonstrated a 1.7-fold downregulation in KISS1 and 9.2-fold downregulation in KISS1r (67). Given the evidence for contrasting roles of KISS1 in relation to inflammation, the

directionality of the hypothesized relationship between KISS1 and inflammation in skeletal muscle is unclear.

Inflammation has also been shown to dysregulate GnRH signaling in the hypothalamus, pituitary, and peripheral tissues. Leukemia inhibitory factor (LIF), a neuroinflammation-induced cytokine, has been shown to reduce spine density, a measure of excitatory synaptic input, of GnRH neurons in the hypothalamus. This inflammation-induced decrease in GnRH neuron spine density was specific to male mice, while protection in females was independent of ovarian estrogens (68). Administration of LPS suppressed gonadotropic function in male rats, likely due to a reduction in absolute responsiveness of GnRH neurons to KISS1 (69). These data suggest that similar to KISS1 signaling, GnRH has a relationship with inflammation in several tissues.

The genetic absence of KISS1 is associated with several phenotypes. In humans, mutations in the KISS1 gene in lead to congenital idiopathic hypogonadotropic hypogonadism, a disorder characterized by incomplete or absent sexual maturation by the age of 18 years accompanied by low levels of circulating testosterone and gonadotropins (70). Whole-body KISS1r genetic knockout mice exhibit reduced lean body mass and higher liver kinase β1 (LKβ1) gene expression in skeletal muscle, with a more robust decrease in males (71). LKβ1 suppresses growth and proliferation through activation of AMP-activated protein kinase (AMPK) and inhibition of mTORC1 (72). However, because the suppression of sex hormones has a significant effect on skeletal muscle development and regenerative capacity (51, 53), the whole-body KISS1r knockout is not sufficient for isolating the roles of endogenous KISS1/GnRH signaling in skeletal muscle. A muscle-specific KISS1 genetic knockout model would be necessary to identify the phenotypes associated with a lack of KISS.

1.9 Conclusions

Chronic inflammation underlies skeletal muscle atrophy and impaired regenerative capacity (6, 13), however, therapies to attenuate inflammation during aging and disease are lacking. In previous research from our laboratory, we found that KISS1 and GnRH were associated with a lower inflammatory susceptibility in skeletal muscle (27). In addition to its roles in maintaining reproductive function and suppressing tumor metastasis, KISS1 signaling has been associated with suppressing inflammation in other tissues (54, 73). KISS1 and GnRH both signal through MAPK pathways, pathways that bridge inflammation and myogenic processes (41). Research that investigates the potential relationship between KISS1 and/or GnRH signaling and inflammation in skeletal muscle offers a novel therapeutic target for chronic inflammation and thus, requires further exploration.

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CHAPTER 2. KISSPEPTIN AND GONADOTROPIN-RELEASING HORMONE SIGNALING IN SKELETAL MUSCLE

2.1 Abstract

Chronic skeletal muscle inflammation contributes to skeletal muscle atrophy and impaired regenerative capacity. Previous research from our laboratory identified that kisspeptin-1 (KISS1) and the gonadotropin-releasing hormone (GnRH) signaling pathway were associated with lower inflammatory susceptibility (i.e., the ability to manage and respond to inflammation) and an improved hypertrophic response in the skeletal muscle. **Objective:** The objectives of this research were to verify the presence of the GnRHr protein in skeletal muscle, elucidate mechanisms of KISS1/GnRH signaling in muscle cells, and investigate the potential relationship between KISS1/GnRH signaling and inflammation in skeletal muscle. **Methods:** To confirm the presence of GnRHr in the skeletal muscle, we extracted the skeletal muscle of ROSA26/GnRHr internal ribosomal entry site Cre (ROSA GRIC [RG]) and wild type (WT) mice and measured β-galactosidase using a commercially available kit. To evaluate downstream GnRH/GnRHr signaling, we used differentiated human primary muscle stem cells (myocytes) and immortalized myoblasts (C2C12 cells) treated with Buserelin, a GnRH analog, and Antide, a GnRH antagonist, and measured canonical GnRHr signaling (i.e. MAPK signaling pathways) with immunoblotting. To evaluate the potential relationship between inflammation and GnRH/GnRHr, NCBI Gene Expression Omnibus (GEO) datasets were searched and analyzed for differential expression of KISS1/GnRH in skeletal muscle. Further, differentiated C2C12 cells were treated with Buserelin and tumor necrosis factor alpha (TNFα), a pro-inflammatory cytokine, and inflammatory signaling was assessed at the mRNA level using RT-PCR analysis. **Results:** We confirmed GnRHr in skeletal muscle; β-galactosidase was detected in RG but not in

WT muscle. In myocytes, extracellular signal-regulated kinase (ERK) signaling responded to Buserelin treatments. The transcription factor cAMP response element-binding protein (CREB) and Jun N-terminal kinase (JNK) responded to Buserelin and Antide treatments. Lastly, KISS1/GnRH expression is downregulated in several inflammatory myopathies; however, Buserelin did not impact TNFα-mediated IL-6 expression in myocytes. **Conclusion:** GnRHr signaling occurs in skeletal muscle, likely through MAPK signaling (i.e., ERK, CREB, JNK), and may be associated with inflammation. Future research is warranted to delineate the relationship between KISS1/GnRH/GnRHr and inflammation.

2.2 Introduction

Chronic inflammation, commonly observed with aging and disease, contributes to skeletal muscle atrophy and impaired regenerative capacity (1, 2). Previous research demonstrated that skeletal muscle inflammatory susceptibility (MuIS), or an impaired ability to regulate and respond to inflammation, is a predictor of failed skeletal muscle regeneration and regrowth following surgery (3, 4). Intriguingly, MuIS, measured in the skeletal muscle, is observed in healthy young males (4) and is also associated with human aging, likely contributing to the adverse structural, metabolic, and functional tissue remodeling that occurs in aged adults (3).

The etiology for MuIS and chronic inflammation in skeletal muscle is unclear, particularly in healthy young adults. Kisspeptin (KISS1) and gonadotropin releasing hormone (GnRH) signaling were identified, in previous research from our lab, as potential therapeutic targets that are associated with MuIS (5). KISS1 was the most highly differentially expressed gene (DEgene) between a group of donor myocytes with (MuIS+) and without (MuIS-) heightened inflammation (downregulated 21.4-fold [MuIS+/MuIS-]) and was a node in the top

network of DEgenes following pathway analysis in Ingenuity Pathways Analysis (IPA). Further, using IPA, the GnRH signaling pathway was identified as a top canonical pathway based on the overrepresentation of DEgenes between MuIS+ and MuIS- cells.

KISS1 is best known for its role in the hypothalamic-pituitary-gonadal axis and activating GnRH neurons (6, 7). Pulsatile GnRH signaling is required for normal reproductive function and stimulates the release of anterior pituitary hormones into the systemic circulation. KISS1 and GnRH are commonly known to signal through the mitogen-activated protein kinase (MAPK) pathways: extracellular-regulated kinase (ERK), Jun N-terminal kinase (JNK), and the downstream transcription factor cAMP response element-binding protein (CREB). These pathways are known to link inflammation and myogenic processes. (8, 9, 10).

Since its discovery, KISS1 has been investigated as a therapeutic target in a number of human cancers (6). KISS1 is downregulated during melanoma progression *in vivo* (11) and KISS1 transfection and constitutive expression suppresses metastasis in human malignant melanoma cells (12). KISS1 is also a potent tumor metastasis suppressor (13). Intriguingly, KISS1 has been shown to inhibit TNFα-mediated activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway in breast cancer cells (14) and dephosphorylate NF-κB in fibrosarcoma cells, resulting in dissociation from the matrix metalloprotease 9 (MMP-9) promoter (15).

While KISS1 has been shown to regulate major proinflammatory pathways in cancer cells, several studies have identified that KISS1/KISS1r signaling itself is dysregulated under inflammatory conditions. An inflammatory challenge with lipopolysaccharide (LPS) decreases KISS1 mRNA expression in the hypothalamus (16) and treatment with an inflammatory stimulus leads to a 1.7-fold downregulation in KISS1 and 9.2-fold downregulation in KISS1r in rat trigeminal ganglion tissue (17). Inflammation also dysregulates GnRH signaling in the

hypothalamus, pituitary, and peripheral tissues. Leukemia inhibitory factor (LIF), a neuroinflammation-induced cytokine, has been shown to reduce spine density, a measure of excitatory synaptic input, of GnRH neurons in the hypothalamus of male mice (18). Finally, administration of LPS suppresses gonadotropic function in male rats, likely due to a reduction in absolute responsiveness of GnRH neurons to KISS1 (19).

The observed relationship between KISS1/GnRH and inflammation in non-muscle cells underscores our findings and thus, supports our desire for further exploration of previous research from our lab. However, KISS1/GnRH protein has never been verified in the skeletal muscle. Further, it is important to establish whether KISS1/GnRH is also expressed in skeletal myopathies. Thus, the purpose of this research was to verify the presence of the GnRHr protein in skeletal muscle, elucidate mechanisms of GnRH signaling in muscle cells, and investigate the potential relationship between KISS1/GnRH signaling and inflammation in skeletal muscle. We hypothesize that GnRHr protein is present in skeletal muscle, GnRH signals through a MAPK pathway in skeletal muscle, and KISS1/GnRH signaling has a relationship with inflammation in skeletal muscle.

2.3 Methods

Ethical approval

This study was approved by the Cornell University Institutional Review Board. Written informed consent was obtained from all human participants. All research involving animals was carried out in accordance with Cornell University's Institutional Animal Care and Use Committee policies.

Mouse skeletal muscle procurement and gene detection assay

To investigate whether GnRHr exists in skeletal muscle, we used a ROSA26/GnRHr internal ribosomal entry site Cre (ROSA26/GRIC) mouse model, which is a β-galactosidase knock-in model, designed to express β-galactosidase in tissues where GnRHr is expressed. Animals were sacrificed at 8 weeks of age using CO_2 inhalation. Skeletal muscle tissue was obtained from the hindlimb of female and male ROSA26/GRIC (n = 5) and C57BL/6J mice (n = 4) at 8 weeks of age. Tissue samples weighing ~30 mg were snap-frozen and stored at -80°C. Samples were pulverized and homogenized using the VWR PowerMAX 200 homogenizer (VWR). Protein was extracted using Radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (PhosSTOP tablets and cOmplete Protease Inhibitor Cocktail tablets, Sigma-Aldrich) and purified by centrifugation at 12,500 x g for 15 min, after which the supernatant was collected, and remaining pellet discarded. Protein levels were quantified using a bicinchoninic assay (BCA Protein Assay Kit, Pierce) with spectrophotometer (Epoch Microplate Spectrophotometer, BioTek). GnRHr was investigated at the protein level using the Galacto-Light Plus™ beta-Galactosidase Reporter Gene Assay System (Thermo Fischer) and analyzed with a SpectraMax M3 luminometer (Molecular Devices). The presence of β-galactosidase was detected by providing a chemiluminescent substrate and measuring luminescence as a representation of β-galactosidase activity. A standard curve was created using exogenous β-galactosidase (Promega) following the Promega protocol.

Human subjects and tissue procurement

Skeletal muscle tissue contains specialized adult stem cells (satellite cells) that reside between the sarcolemma and basal lamina. Satellite cells can be harvested from human muscle tissue and cultured. Cultured satellite cells, from human donors (muscle progenitor cells [*h*MPCs]), can be induced to differentiate (myocytes) and form multinucleated myotubes.

Because *h*MPCs and the myocytes retain properties of the *in vivo* state, they are an optimal model to study not only myogenesis, but also factors that impact skeletal muscle health.

*h*MPCs from healthy, ambulant young male (n = 5) and female (n = 5) donors were used to address research objectives. Potential participants were recruited from the Tompkins County, New York area via flier and web-based announcements. All individuals completed a comprehensive health history and physical activity questionnaire and were independently ambulatory and cognitively intact as determined by the examining nurse practitioner. Individuals were excluded for contagious infections and any chronic end-stage disease expected to limit life-expectancy to less than one year, induce anorexia, or restrict physical activity. Individuals with seated resting systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg and individuals receiving anabolic (e.g., growth hormone, insulin-like growth factor 1 [IGF-1]) therapy were also excluded.

Skeletal muscle biopsies were performed by a nurse practitioner in the Human Metabolic Research Unit (HMRU) at Cornell University. After an overnight fast, subjects reported to the HMRU, vitals (i.e., heart rate and blood pressure) were measured, and subjects rested in the supine position. A percutaneous needle biopsy of the vastus lateralis was taken using a 5.0 mm Bergstrom biopsy needle with suction under local anesthetic (1% lidocaine). Tissue was quickly blotted with sterile gauze and visible adipose and connective tissues were removed. Approximately 75-100 mg of the total muscle biopsy was stored in Gibco® Hibernate®A (Thermo Fisher Scientific) at 4°C until tissue disassociation was performed.

Purified, human primary muscle progenitor cells

Biopsy tissue in Gibco® Hibernate®A (Thermo Fisher Scientific) was minced and washed via gravity with Ca-Mg free D-PBS. Minced and washed tissue was then disassociated in

digest medium (2 mg/mL Collagenase D [Roche] in low-glucose DMEM). After 30 min in digest medium, fresh digest medium and Dispase (Sigma) were added. The disassociating pellet was titurated until a uniform slurry was achieved. Growth medium ([GM] Hams F12 + 20% FBS + 5 ng/mL bFGF [Promega] + 1% Pen/Strep [Gibco]) was added to the slurry and passed through a 70 μm cell strainer into a sterile tube. The cell suspension was centrifuged. The pellet was resuspended in Recovery® freezing medium (Gibco) and cryopreserved at -80 °C. Thawed cells were seeded on a type-I collagen coated culture dish (initial cell confluency ~15%). Cells were cultured at 37 °C under 5% CO_2 atmospheric conditions. After 24 h, the GM was replaced with fresh GM and further replenished every 48 h. Once cells reached ~70% confluency, they were removed from the plate using 0.25% Trypsin-EDTA and passaged. Cells were expanded to passage four, then cryopreserved in 10% DMSO + GM.

Approximately 1-1.5 million cells were labeled with fluorescently-conjugated antibodies specific for myoblast cell surface antigens CD56 (NCAM; PE-Cy7-conjugated) and CD29 (β1-integrin; AlexaFluor488-conjugated) and the viability stain 7-Aminoactinomycin D (7AAD) (187). Individual samples yield 150,000 – 800,000 viable CD56+/CD29+ *h*MPCs per 1 million cells sorted using a BD FACS Aria™ Fusion flow cytometer. Sorted CD56+/CD29+ cells were expanded in culture by passaging twice and then used in experiments; all *in vitro* experiments were performed using sorted cells at passage six.

GnRH/GnRHr functional signaling pathway in human primary myocytes

Sorted *h*MPCs were seeded at a density of approximately 10%. Throughout proliferation, GM was changed every 48 h. At ~70% confluence, *h*MPCs were switched to differentiation media ([DM] Ham's F12 [Gibco] supplemented with 2% heat inactivated equine serum]. At 5 d post-differentiation, myocytes were serum-starved for 4 h during which time they were treated

with DM + 10 nM Buserelin (Buserelin acetate salt, Sigma-Aldrich). Buserelin is a GnRH agonist. This concentration of Buserelin has been used in culture settings in previous research. Cell lysate was collected at time 0 min, 5 min, 15 min, 30 min, 60 min, and 120 min after DM treatments with RIPA buffer supplemented with phosphatase and protease inhibitors (PhosSTOP tablets and cOmplete Protease Inhibitor Cocktail tablets, Sigma-Aldrich) and protein was purified by centrifugation at 12,500 x g for 15 min, after which the supernatant was collected, and remaining pellet discarded. Protein levels were quantified using a bicinchoninic assay (BCA Protein Assay Kit, Pierce) with a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek).

GnRH/GnRHr functional signaling pathway in C2C12 myocytes

C2C12 cells (ATCC) were rapidly thawed from storage at -80°C and seeded onto 10 cm plates in C2C12 growth media ([CGM] Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum) to reduce variability introduced by the freeze-thaw process. CGM was changed at 24 h. At approximately ~70% confluency, cells were removed from the plate using 0.25% Trypsin-EDTA, passaged, and seeded at a density of approximately 10% in CGM. At ~70% confluence, cells were switched to C2C12 differentiation media ([CDM] Dulbecco's Modified Eagle Medium supplemented with 2% heat inactivated equine serum). At 5 d post-differentiation, C2C12 myocytes were serum-starved for 4 h during which time they were treated with CDM; CDM + 10 nM Buserelin (Buserelin acetate salt, Sigma-Aldrich); or CDM +100 nM Antide (Sigma-Aldrich) + 10 nM Buserelin (Antide was administered 30 min prior to Buserelin). Antide is a GnRH antagonist. This concentration of Antide has been used in culture settings in previous research. Cell lysate was collected at time 0 min, 5 min, 15 min, 30 min, 60 min, and 120 min after CDM treatments with RIPA buffer supplemented with phosphatase and protease

inhibitors (PhosSTOP tablets and cOmplete Protease Inhibitor Cocktail tablets, Sigma-Aldrich). Protein was purified by centrifugation at 12,500 x g for 15 min, after which the supernatant was collected, and remaining pellet discarded. Protein levels were quantified using a bicinchoninic assay (BCA Protein Assay Kit, Pierce) with a spectrophotometer (Epoch Microplate Spectrophotometer, BioTek).

Western blot analysis

Western blot analysis was used to determine protein levels on known GnRH/GnRHr functional signaling pathways, in human myocytes and differentiated C2C12 myocytes. Purified total protein extracts were diluted with double distilled water (ddH2O) and Laemmli loading buffer to standardize protein content to 12 ug, loaded in 10% SDS gels, and transferred to biological membranes overnight at 4 °C. Membranes were blocked for 1 h in a chemiluminescent blocking agent (Immobilon Block, Millipore). Primary antibodies (Cell Signaling Technology) against p-ERK1/2 (1:1,000), total ERK1/2 (1:1000), p-JNK (1:1000), JNK (1:1000), p-CREB (1:1000), total CREB (1:1000), and α-tubulin (1:1000) were diluted in blocking agent (Immobilon Block, Millipore). Membranes were incubated in primary antibody solutions overnight at 4° C on a rocker. Membranes were washed in tris-buffered saline supplemented with Tween 20 (TBST) three times on a rocker, for 5 min each, before incubation in secondary antibody. Horseradish peroxidase-conjugated secondary rabbit antibodies were diluted 1:100,000 and mouse antibodies 1:125,000 in blocking agent. Membranes were incubated in secondary antibody for 1 h at room temperature on a rocker and washed again as indicated above. Blots were developed in imaging solution (SuperSignal West Femto Stable Peroxide Buffer and Luminol/Enhancer Solution, Thermo Scientific) and analyzed with the ChemiDoc MP Imaging System (Bio Rad). Prior to reuse, membranes were stripped for 5 min with Restore PLUS

Western Blot Stripping Buffer (Thermo Scientific) and checked for remaining bound antibody using the ChemiDoc before subsequent blocking. All blots were normalized to the housekeeper α-tubulin.

KISS/GnRH gene expression in GEO Datasets

The National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) Datasets were searched for conditions in which KISS1, KISS1r, GnRH, or GnRHr was differentially expressed in skeletal muscle tissue (Figure 1). Search terms included "muscle, skeletal "[MeSH Terms] OR skeletal muscle [All Fields]. GEO2R software was used to run statistical analyses. Two or more groups were compared to identify differentially expressed genes (DEgenes) across conditions or treatment groups. Comparisons were performed using GEOquery and Linear Models for Microarray Analysis (limma), which are R packages from the Bioconductor project (20). Bioconductor is an open source software project that aids in analyses of high-throughput genomic data. GEOquery parses data into R structures that are usable to other R packages. Limma is the most widely used statistical test to identify DEgenes. Results were collected from a table of DEgenes arranged in order of statistical significance. All p-values presented were adjusted for false discovery rate using the Benjamini & Hochberg procedure, the most common multiple-testing correction for microarray data. The Benjamini & Hochberg adjustment provides a balance between detecting statistically significant differences and avoiding false positives. Graphs were generated using the “Profile Graph” feature and Microsoft Excel.

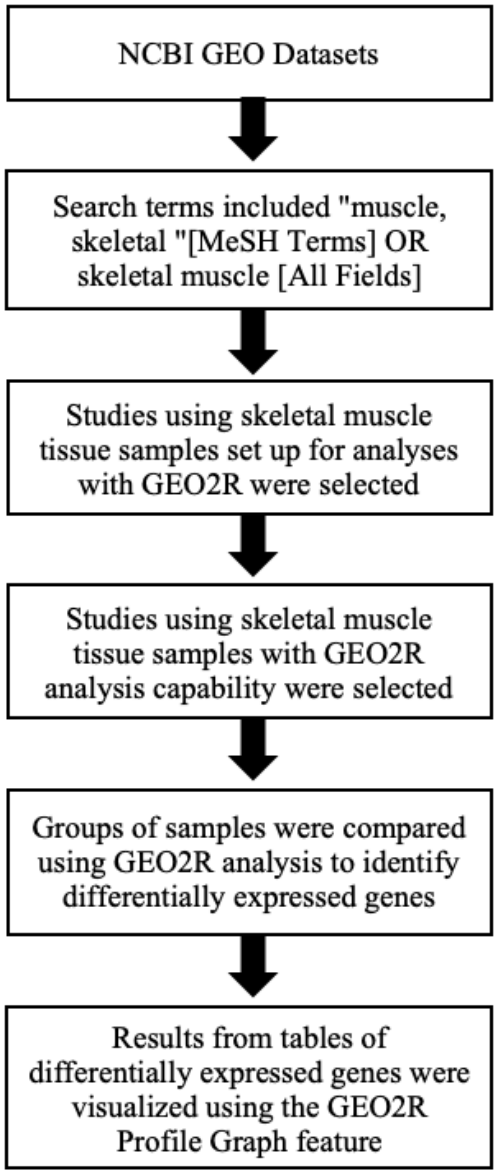


Figure 1. Literature Search of NCBI GEO Datasets.

GnRH signaling and inflammatory cytokine gene expression

Sorted *h*MPCs from young female (n = 5) and young male (n = 5) donors were seeded at a density of approximately 10%. Throughout proliferation, GM was changed every 48 h. At ~70% confluence, *h*MPCs were switched to DM and differentiated for 5 days (myocytes). Myocytes were treated for 48 h with DM (control); DM + 10 ng/mL TNFα; DM + 10 nM

Buserelin; or DM + 10 ng/mL TNFα + 10 nM Buserelin. 15 min prior to harvest, fresh treatments were administered to myocytes. RNA was harvested using TRK lysis buffer (Omega) following manufacturer's instructions. Total RNA was purified using an EZNA total RNA kit (Omega) following manufacturer's instructions. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time, quantitative PCR (RT-PCR) was performed using the LightCycler 480 system (Roche) with the TaqMan Fast Advanced Master Mix and TaqMan® Gene Expression Assays (Applied Biosystems) to identify differences in gene expression of *IL-6. 18S* (Hs99999901_s1) was used as the housekeeper.

Statistical Analyses

Protein levels of GnRHr signaling and mRNA levels of inflammatory cytokines, in myocytes, were analyzed using a two-way analysis of variance (ANOVA) with Tukey post hoc tests if the interaction term was significant. All statistical tests on mRNA data were performed using the delta Cp (dCP) values for *IL-6* and *18s*. All statistical analyses of experimental results were completed using GraphPad Prism4. All values are reported as mean ± s.d.. Significance was set at $p < 0.05$.

2.4 Results

GnRHr gene detection in skeletal muscle

The ROSA26/GRIC mouse model is a β-galactosidase knock-in, designed to express β-galactosidase in tissues where GnRHr is expressed. β-galactosidase enzyme activity was detected in the skeletal muscle of the ROSA26/GRIC mice but not in the C57BL/6J (WT) mice (Figure 2). This result validates the presence of GnRHr in skeletal muscle and confirms our hypothesis that GnRHr protein exists in skeletal muscle. While we could not determine statistical

differences between males and females for GnRHr, the ROSA26/GRIC males appeared to have greater β-galactosidase activity compared to the ROSA26/GRIC females (Figure 2).

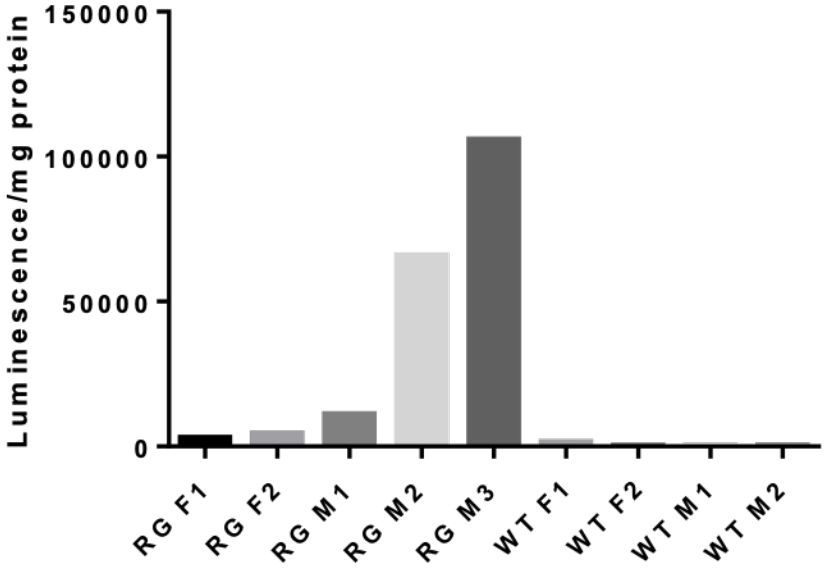


Figure 2. GnRHr Validation. β-galactosidase enzyme activity was measured using luminescence and normalized to total protein in skeletal muscle harvested from female (F) and male (M) ROSA26/GRIC (RG) and C57BL/6J (WT) mice. Data are presented as individual mice.

GnRH/GnRHr functional signaling pathway in skeletal muscle

To further validate the presence and function of GnRH/GnRHr in skeletal muscle, we measured canonical signaling, through MAPK signaling pathways, in human primary myocytes treated with Buserelin, a GnRH analog. Buserelin treatment caused a transient, upward spike in activation of the ERK1 signaling pathway over time, with a decline in p-ERK at 2 h ($p= 0.19$, Figure 3). However, there was large variation in activity among cultures, which is common with human primary cells; thus, we did not observe a statistically significant difference in Buserelin-induced activation of ERK. Therefore, we repeated the time course experiment using C2C12 myocytes. In C2C12 myocytes, there was no effect of Buserelin alone, over time, on p-ERK

(Figure 4A-B), p-CREB (Figure 5A-B), or p-JNK (Figure 6A-B). We did observe a trend in JNK activation ($p = 0.08$, Figure 6A-B) over time; however, there was large variation among replicates. To verify any activation of MAPK signaling was induced by Buserelin, additional C2C12 myocytes were treated with Antide, a GnRH antagonist that binds but does not activate GnRHr, 30 min before Buserelin treatment. Because Antide binds the GnRHr, it inhibits Buserelin from binding. Treatment with Buserelin caused a significant activation of CREB at 60 min ($p = 0.0002$, Figure 5C). Intriguingly, there was no effect of Buserelin on CREB activation when cells were pretreated with Antide ($p = 0.71$, Figure 5D). Further, JNK activation was lower across time in cultures treated with Antide prior to treatment with Buserelin compared to Buserelin alone; however, this was not statistically significant.

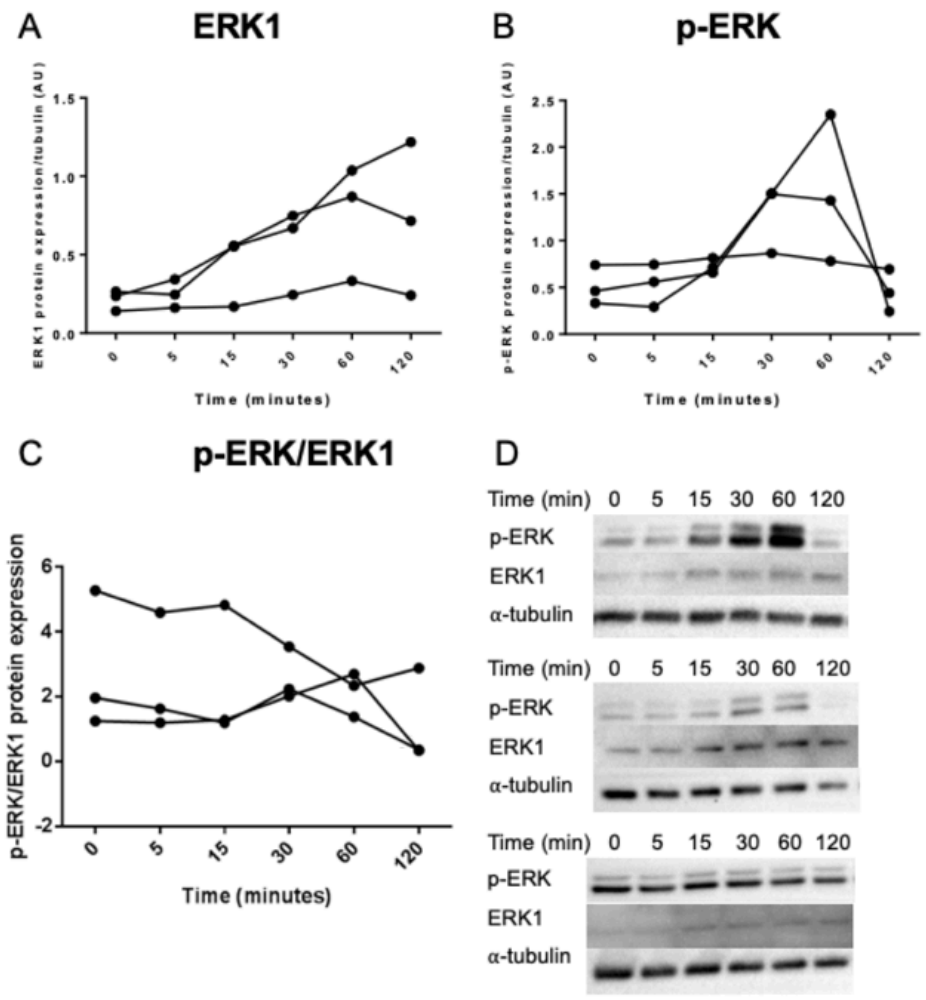


Figure 3: Western Blot Analysis of ERK in Myocytes. (A) Total ERK1 and (B) p-ERK protein levels were measured by Western blot analysis of proteins obtained from human primary myocytes at 6 time points after treatment with Buserelin (n = 3). (C) The ratio of p-ERK/ERK1 protein expression was calculated to assess active ERK1 signaling. (D) Western blots of p-ERK, total ERK1, and α-tubulin at 6 time points using protein from 3 young male donors. All blots were normalized to α-tubulin. Data are presented as individual donors.

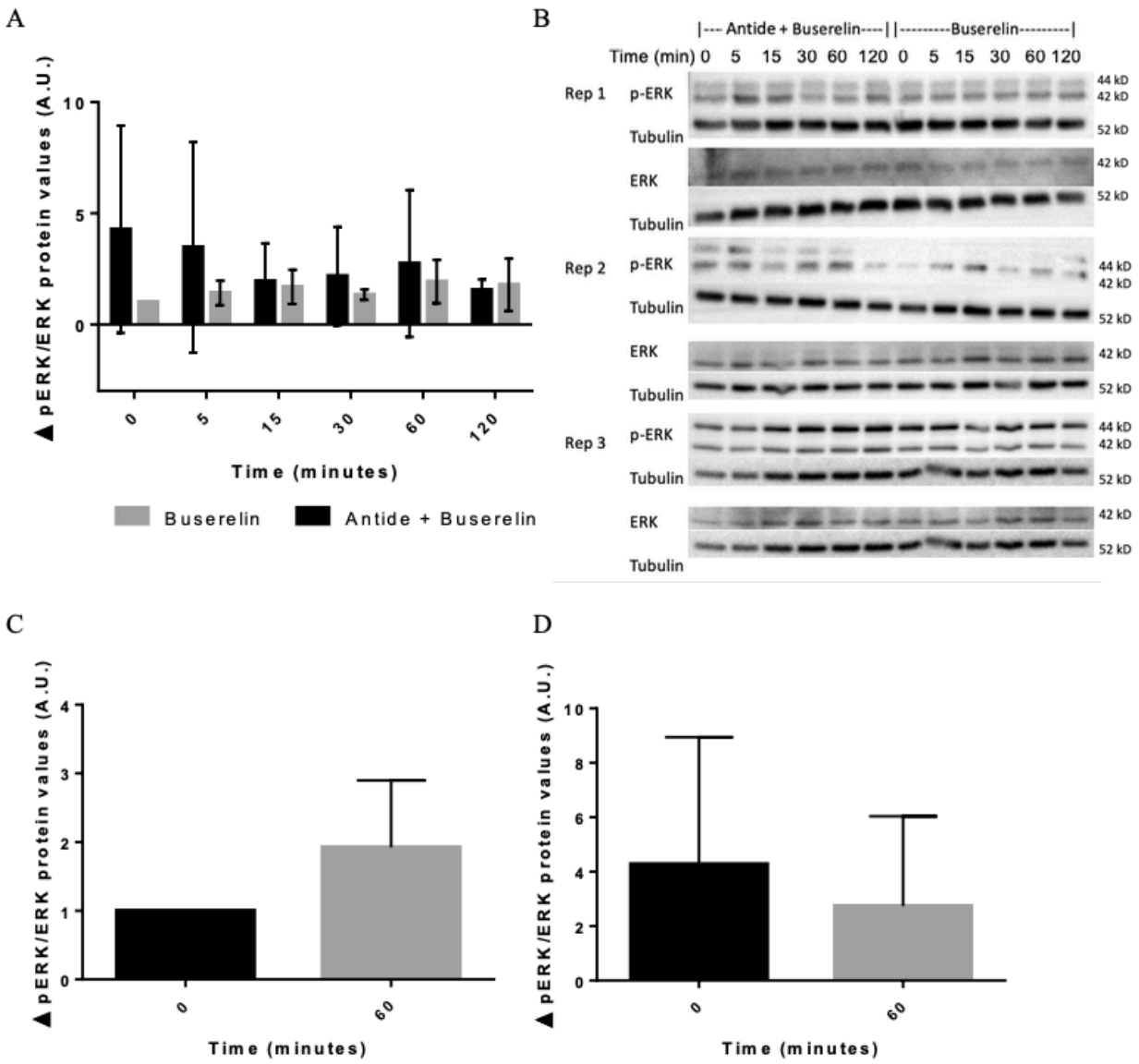


Figure 4. Western Blot Analysis of ERK in C2C12 Myocytes. (A) **Δ** p-ERK/ERK protein value (A.U.) with Antide + Buserelin or Buserelin treatment (B) p-ERK/ERK Western blot analysis. (C) **Δ** p-ERK/ERK protein value (A.U.) without Buserelin treatment (Time 0 min) and with Buserelin treatment (Time 60 min). (D) **Δ** p-ERK/ERK protein value (A.U.) 30 min after Antide treatment (Time 0 min) and 60 min after Buserelin treatment with Antide administration 30 min prior to Buserelin (Time 60 min). Protein levels were measured by Western blot analysis of proteins obtained from C2C12 cells at 6 time points following treatment with Antide + Buserelin (n = 3) or Buserelin (n = 3). All blots were normalized to α-tubulin and further normalized to Time 0 of the Buserelin treatment. A two-way ANOVA (treatment-by-time) and t tests comparing Time 0 min and Time 60 min were performed. All values are reported as mean ± s.d. Significance was determined at $p < 0.05$. Effect of treatment $p = 0.15$. Effect of Buserelin at 60 min $p = 0.17$. Effect of Antide + Buserelin at 60 min $p = 0.67$.

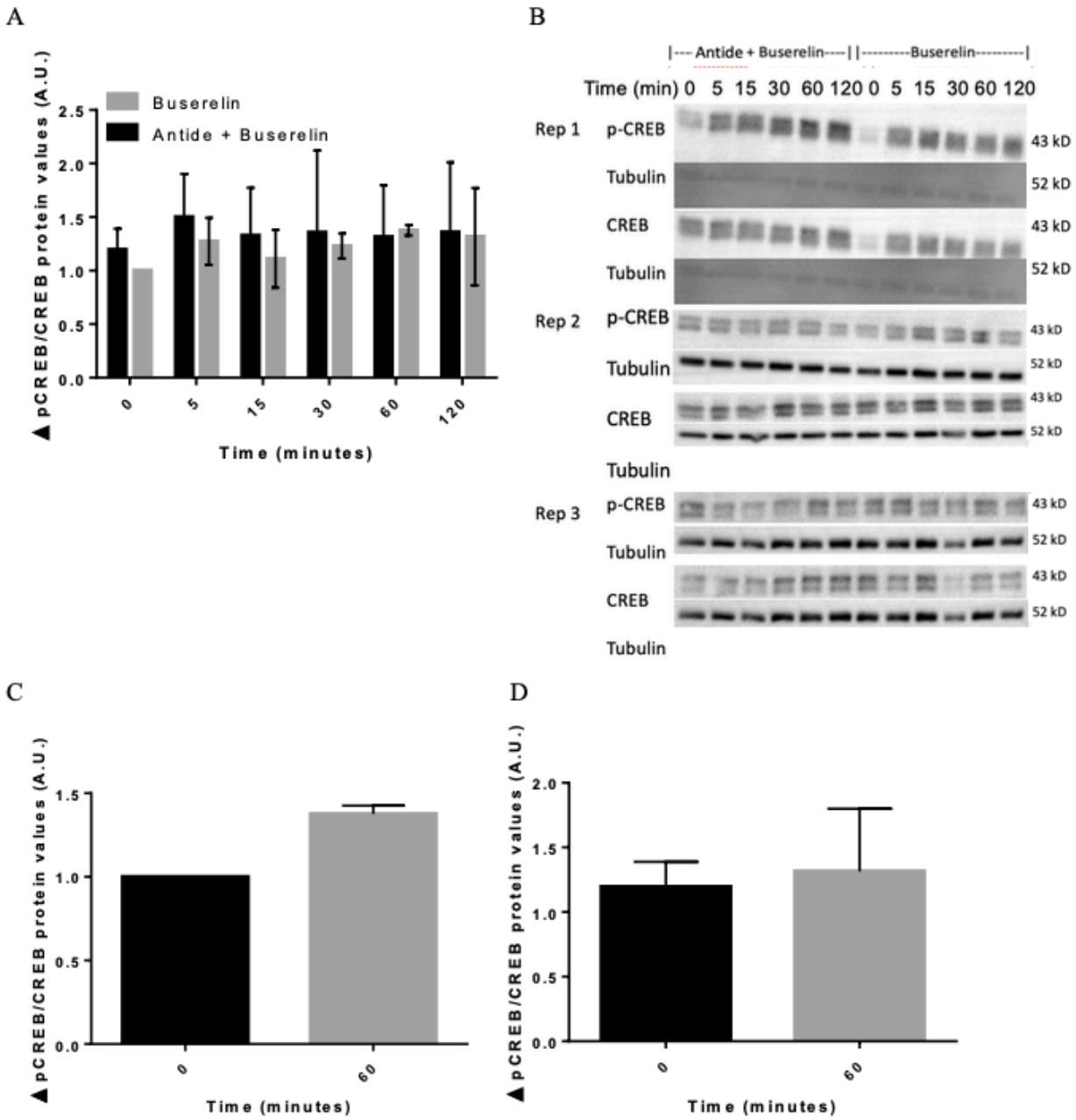


Figure 5. Western Blot Analysis of CREB in C2C12 Myocytes. (A) **Δ** p-CREB/CREB protein value (A.U.) with Antide + Buserelin or Buserelin treatment (B) p-CREB/CREB Western blot analysis. (C) **Δ** p-CREB/CREB protein value (A.U.) without Buserelin treatment (Time 0 min) and with Buserelin treatment (Time 60 min). (D) **Δ** p-CREB/CREB protein value (A.U.) 30 min after Antide treatment (Time 0 min) and 60 min after Buserelin treatment with Antide administration 30 min prior to Buserelin (Time 60 min). All blots were normalized to α-tubulin and further normalized to Time 0 of the Buserelin treatment. A two-way ANOVA (treatment-by-time) and t tests comparing Time 0 min and Time 60 min were performed. All values are reported as mean ± s.d. Significance was determined at $p < 0.05$. Effect of treatment $p = 0.37$. Effect of Buserelin at 60 min $p = 0.0002$. Effect of Antide + Buserelin at 60 min $p = 0.71$.

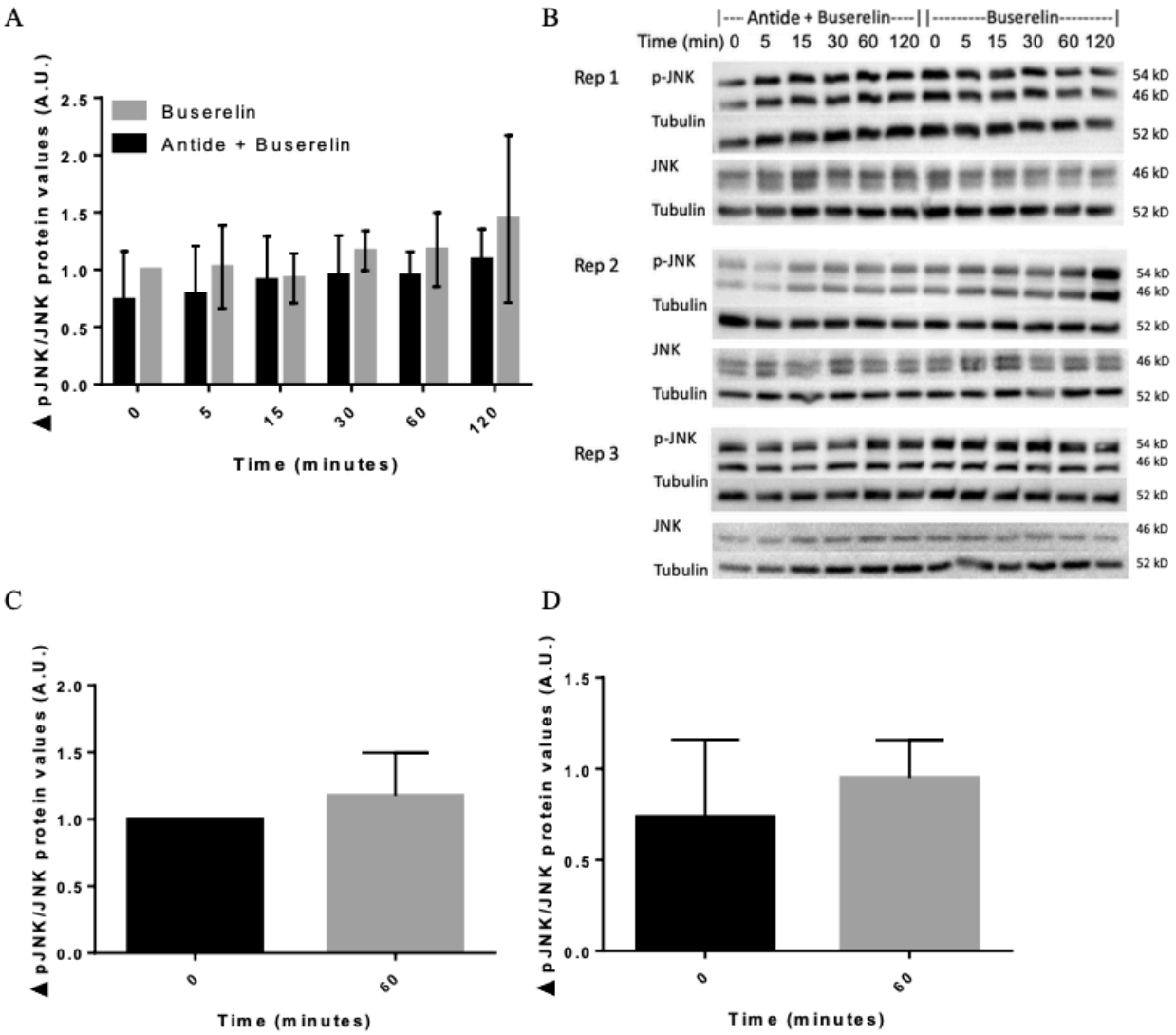


Figure 6. Western Blot Analysis of JNK in C2C12 Myocytes. (A) **Δ** p-JNK/JNK protein value (A.U.) with Antide + Buserelin or Buserelin treatment (B) p-JNK/JNK Western blot analysis. (C) **Δ** p-JNK/JNK protein value (A.U.) without Buserelin treatment (Time 0 min) and with Buserelin treatment (Time 60 min). (D) **Δ** p-JNK/JNK protein value (A.U.) 30 min after Antide treatment (Time 0 min) and 60 min after Buserelin treatment with Antide administration 30 min prior to Buserelin (Time 60 min). All blots were normalized to α-tubulin and further normalized to Time 0 of the Buserelin treatment. A two-way ANOVA (treatment-by-time) and t tests comparing Time 0 min and Time 60 min were performed. All values are reported as mean ± s.d. Significance was determined at $p < 0.05$. Effect of treatment $p = 0.08$. Effect of Buserelin at 60 min $p = 0.40$. Effect of Antide + Buserelin at 60 min $p = 0.48$.

KISS/GnRH gene expression in GEO Datasets

To determine whether KISS/GnRH gene expression is downregulated in the skeletal muscle of patients with diagnosed myopathies, we conducted secondary analyses of NCBI GEO datasets. We determined that the gene expression of KISS1, KISS1r, GnRH, and GnRHr was significantly reduced in myopathies including juvenile dermomyositis, Duchenne muscular dystrophy, amyotrophic lateral sclerosis, facioscapulohumeral dystrophy, tibial muscular dystrophy, and myotonic dystrophy type 2. KISS1 was downregulated in the skeletal muscle of untreated girls with juvenile dermomyositis vs. healthy controls **(**Figure 7**) (**21). GnRH2 and GnRHr expression were reduced in Duchenne muscular dystrophy **(**Figure 8), amyotrophic lateral sclerosis (Figure 9), and facioscapulohumeral dystrophy (Figure 10) compared to control skeletal muscle (22, 23). KISS1 and GnRH2 gene expression were not significantly different in myotonic dystrophy type 2 ($p > 0.05$, Figure 11) vs. control skeletal muscle (24). Gene expression of GnRH1, but not GnRHr trended downward in tibial muscular dystrophy vs. control skeletal muscle ($p = 0.058$, Figure 12**)** (24).

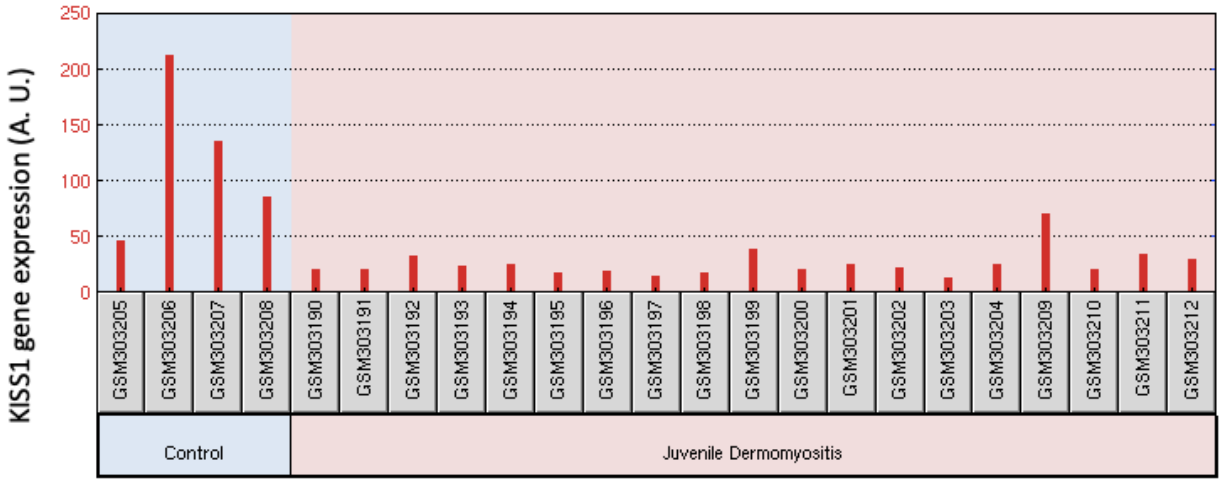


Figure 7. KISS1 Gene Expression in Juvenile Dermomyositis. KISS1 ($p = 1.25e-06$) gene expression in control ($n = 4$) vs. untreated (juvenile) dermomyositis ($n = 19$) skeletal muscle.

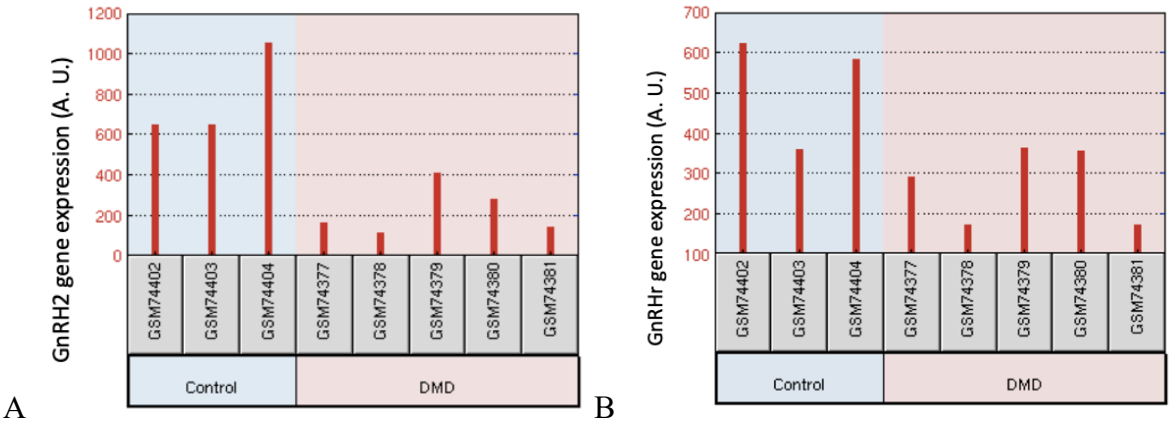


Figure 8. GnRH2 and GnRHr Gene Expression in Duchenne Muscular Dystrophy (DMD). (A) GnRH2 (p = 2.04e-03) and (B) GnRHr (p = 0.024) gene expression in control (n = 3) vs. Duchenne muscular dystrophy (DMD) (n = 5) skeletal muscle.

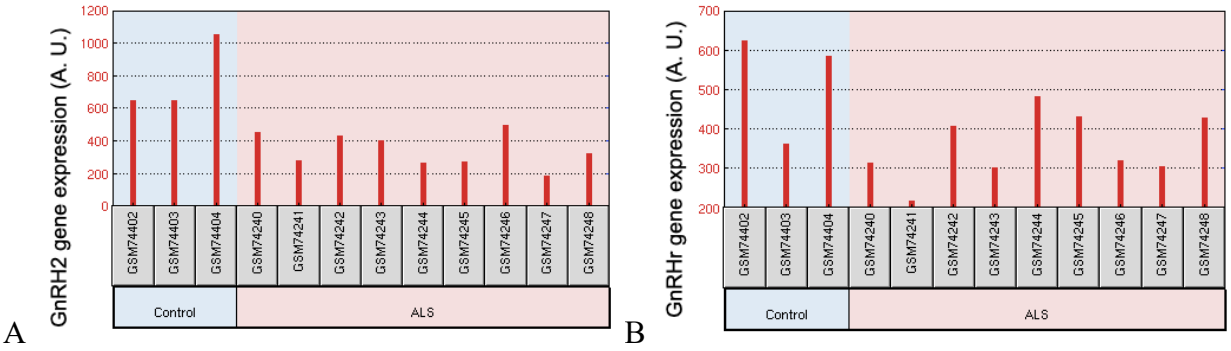


Figure 9. GnRH2 and GnRHr Gene Expression in Amyotrophic Lateral Sclerosis (ALS). (A) GnRH2 (p = 1.26e-03) and (B) GnRHr (p = 0.049) gene expression in amyotrophic lateral sclerosis (ALS) (n = 9) vs. control (n = 3) skeletal muscle.

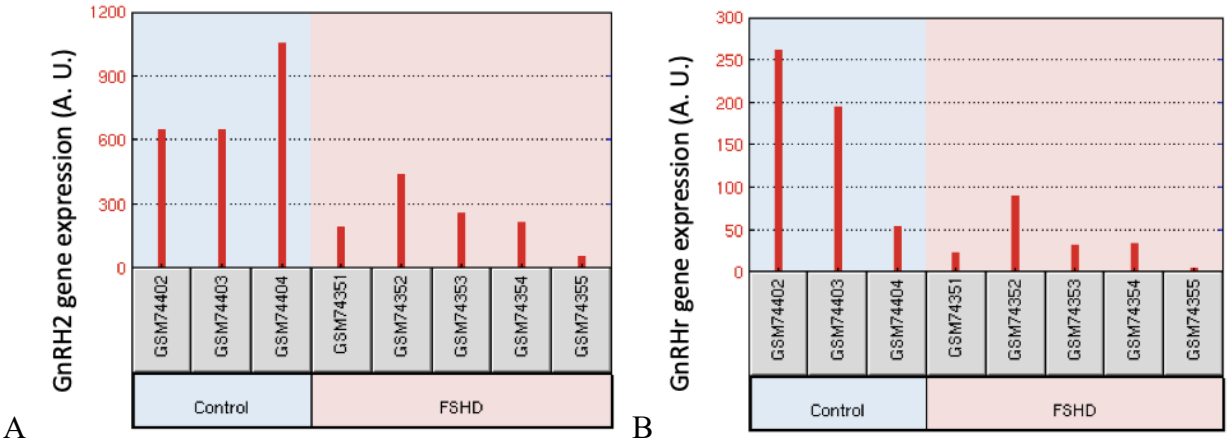


Figure 10. GnRH2 and GnRHr Gene Expression in Facioscapulohumeral Dystrophy (FSHD). (A) GnRH2 (p = 9.40e-03) and (B) GnRHr (p = 0.02) gene expression in control (n = 3) vs. facioscapulohumeral dystrophy (FSHD) (n = 5) skeletal muscle.

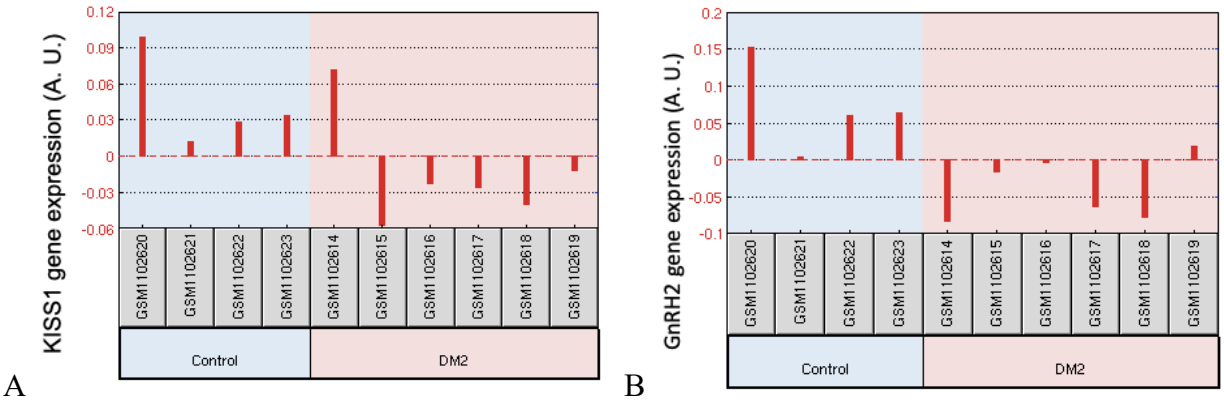


Figure 11. KISS1 and GnRH2 Gene Expression in Myotonic Dystrophy Type 2 (DM2). (A) KISS1 (p = 0.55) and (B) GnRH2 (p = 0.25) gene expression in control (n = 4) vs. myotonic dystrophy type 2 (DM2) (n = 6) skeletal muscle.

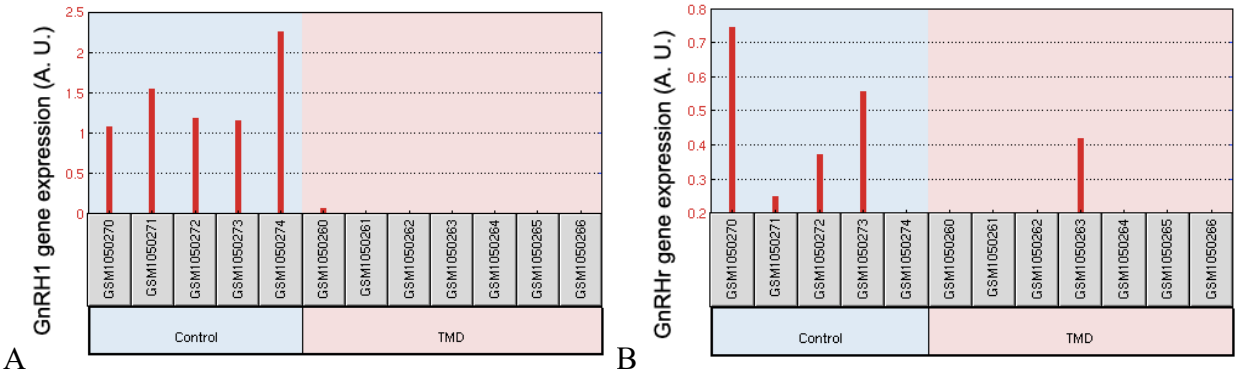


Figure 12. GnRH1 and GnRHr Gene Expression in Tibial Muscular Dystrophy (TMD). (A) GnRH1 (p = 0.058) and (B) GnRHr (p = 0.93) gene expression in tibial muscular dystrophy (TMD) (n = 7) vs. control (n = 5) skeletal muscle.

To determine whether KISS1 signaling is negatively regulated with inflammation in tissues other than skeletal muscle, we conducted secondary analyses of available GEO datasets that are associated with heightened inflammation. Gene expression of KISS1r was downregulated in whole liver tissue of mice that underwent cecum ligation and puncture to produce sepsis vs. control mice (Figure 13) (25). KISS1r was downregulated in breast cancer cells transfected with small interfering RNA (siRNA) targeting Inhibitor of nuclear factor kappa-

B kinase subunit beta (IKKβ), a key component of the NF-κB inflammatory signaling pathway (Figure 14) (26).

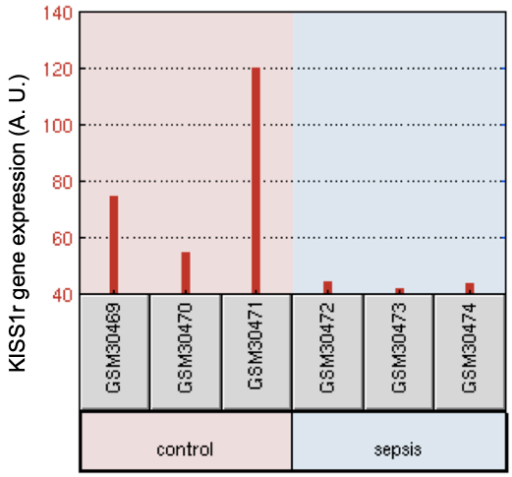


Figure 13. KISS1r Gene Expression in Sepsis. KISS1r gene expression (p = 0.03) in whole liver tissue of control (sham, n = 3) and septic (n = 3) mice.

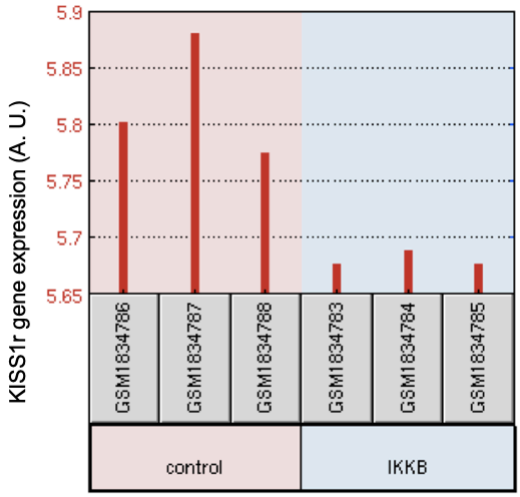
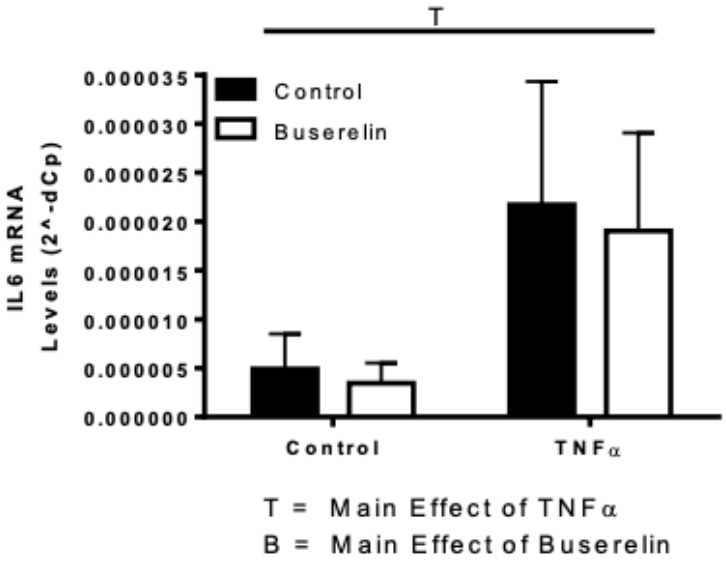


Figure 14. KISS1r Gene Expression and Inflammation in Breast Cancer Cells. KISS1r gene expression (p = 0.03) in breast cancer cells (control) (n = 3) vs. breast cancer cells transfected with siRNA targeting IKKβ (n = 3).

GnRH signaling and inflammatory cytokine gene expression

To explore the relationship between GnRH signaling and inflammation in skeletal muscle, we measured IL-6 gene expression in myocytes from young female (n = 5) and young male (n = 5) donors cultured in DM (control), TNFα, Buserelin, or TNFα + Buserelin for 48 h. As expected, treatment with TNFα significantly upregulated IL-6 mRNA levels in both males and females, while treatment with Buserelin had no effect under basal or inflammatory conditions (Figure 15).

A



B

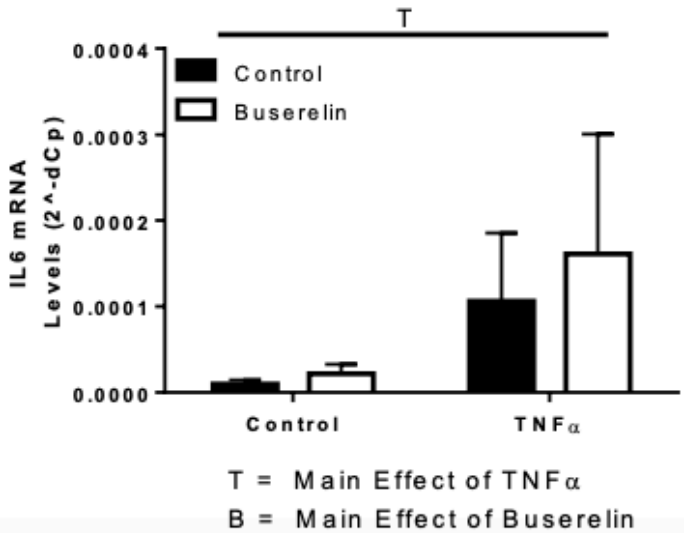


Figure 15. Inflammatory Cytokine mRNA Levels in Myocytes. IL-6 mRNA expression in myocytes from (A) young female (n = 5) and (B) young male donors (n = 5) treated with differentiation media control, TNFα, Buserelin, or TNFα + Buserelin given together for 48 h. IL-6 expression values were normalized to the housekeeper gene 18S. Two-way ANOVA were performed (Buserelin-by-TNFα) on the delta Cp (dCP) values. Significance was set at $p < 0.05$. T = significant effect of TNFα.

2.5 Discussion

Our research is the first to identify KISS1/GnRH in the skeletal muscle and further, this research identifies a potential novel link between heightened inflammation and KISS1/GnRH signaling. In addition to its role in the hypothalamic-pituitary-gonadal axis, KISS1 has been linked to inflammation in various non-muscle tissues and cell types. KISS1 inhibits the TNFα-mediated activation of the proinflammatory NF-κB pathway in breast cancer cells (14), reduces NF-κB binding to the promoter in fibrosarcoma cells (15), and inhibits cancer metastasis in several other cancers (6, 12, 27). To our knowledge, KISS1 signaling has not been previously identified in skeletal muscle. Interestingly, GnRH signaling emerged as a top canonical pathway based on DEgenes identified between our MuIS+ and MuIS- myocyte cultures as well as between individuals who exhibited no response or an extreme hypertrophic response to resistance exercise training (28, 29). Intriguingly, those who had no response to resistance exercise training also had heightened expression of inflammation-related genes. While GnRH signaling has been identified in numerous extra-pituitary tissues, GnRH in skeletal muscle is a novel pathway that warranted further investigation.

GnRHr was detected at the protein level with a β-galactosidase chemiluminescent reporter gene detection assay in skeletal muscle of ROSA26/GRIC but not C57BL/6J mice, validating the presence of GnRHr in skeletal muscle. This result revealed potential sex

differences, as male ROSA26/GRIC mice appeared to have increased levels of GnRHr compared to ROSA26/GRIC females. A recent study showed that an inflammation-induced reduction in spine density, a measure of excitatory synaptic input, of GnRH neurons in the hypothalamus occurs in male but not female mice (18). Further, administration of LPS suppressed gonadotropic function in male rats, likely due to a reduction in absolute responsiveness of GnRH neurons to KISS1 (19). Aside from inflammation, in the reproductive system, GnRH-induced ERK activation is required for female, but not male, fertility (8). Considering the well-recognized sexual dimorphism of KISS1 and GnRH signaling in other systems and evidence suggesting sex differences in response to inflammation, it is feasible the relationship between KISS1 and/or GnRH signaling and inflammation may involve sex differences in skeletal muscle.

GnRH/GnRHr signaling through CREB may be functional in skeletal muscle; we found that the addition of Antide, a competitive inhibitor that binds GnRHr, diminished CREB activation at 60 min compared to Buserelin alone. Similar results were also observed with JNK and ERK activation, however the variation across replicates did not allow for statistical significance. The inconsistent and insufficient MAPK signaling we observed with Buserelin treatment could be due to the rapid and transient nature of these proteins. It is possible that activation occurred but was overlooked using only the specified time points. For example, Caunt et al. (2012) found a maximum response of GnRH-induced ERK1/2 signaling at 5 min followed by rapid reduction close to basal levels at 60 min in a GnRHr overexpression model of HeLa cells (30). In addition to inflammatory cytokines, the MAPK pathways are stimulated by growth factors, mitogens, and environmental stressors. (31). In skeletal muscle, MAPKs are activated by IGF-1, fibroblast growth factor (FGF), and numerous other signals (32, 33, 34). Due to the complex nature of MAPK signaling in skeletal muscle cells, the Western blot analysis method may be limited in its ability to detect MAPK signaling. Because Antide blunted activation by

Buserelin, we are fairly confident that GnRHr signaling in the muscle acts via CREB and JNK, however follow-up studies to confirm this signaling are necessary.

Results from searching NCBI GEO datasets further support the notion that there is a relationship between KISS1/GnRH and inflammation in the skeletal muscle; KISS1/GnRH signaling may be downregulated in various myopathies. However, this theory was not supported in myocyte culture when we treated myocytes with TNFα + Buserelin for 48 h. Buserelin had no significant effect on TNFα-mediated IL-6 expression in myocytes from males or females. Similar to the human myocyte and C2C12 myocyte experiments using Western blot analysis to determine GnRH-induced MAPK signaling in skeletal muscle, these results may have excluded the transient length of time that Buserelin alters TNFα-mediated IL-6 expression. It is also possible that the directionality of the relationship may be contrary to the original hypothesis; KISS1/GnRH signaling in skeletal muscle may be diminished in response to inflammation. However, this theory is challenging to test due to the insufficiency of high-quality antibodies for KISS1, GnRH, and GnRHr.

Other hormones central to the mammalian reproductive system have been shown to also regulate skeletal muscle homeostasis and regeneration under basal and inflammatory conditions. Oxytocin, best known for its role in lactation, parturition and behavior, is required for skeletal muscle homeostasis and successful regeneration (35). Plasma levels of oxytocin decline with age and a genetic disruption of oxytocin is associated with premature muscle atrophy and impaired muscle function. Estrogen, the primary mammalian female sex hormone, is also known to stimulate muscle repair and regeneration, including satellite cell activation and proliferation. In female mice, prolonged estrogen deficiency leads to a shift towards glycolytic myofiber types, impaired SC function, diminished regenerative capacity, and muscle atrophy (36). Estrogen alleviates the loss of skeletal muscle mass associated with sepsis and blunts sepsis-induced

hypothalamic inflammation and dysregulation of hypothalamic neuropeptides (37). Estrogen lowered training-induced muscle damage in post-menopausal women (38). Intriguingly, our previous research showed that β-estradiol, the predominant estrogen hormone, was predicted to be activated as an upstream regulator in MuIS-, suggesting that β-estradiol was elevated in the skeletal muscle of MuIS- individuals prior to the observed increase in KISS1 gene expression. In the hypothalamus, estrogen exerts both positive and negative feedback regulation on GnRH release through KISS1 neurons (39). It is possible that estrogen regulates KISS1 expression in skeletal muscle.

Limitations

KISS1 signals through GnRH, however, KISS1 protein or gene expression was not examined in this study due to time constraints and limitations of commercial antibodies currently available. It is possible that the association between KISS1 and inflammation in skeletal muscle is independent of GnRH. There are other potential pathways by which KISS1 and/or GnRH signals to regulate inflammation in skeletal muscle.

2.6 Conclusion

These results confirm that GnRHr protein is present and functional in skeletal muscle. Further, these results demonstrate that KISS1/GnRH have a relationship with inflammation that is impacted with inflammatory susceptibility and myopathies. Future research should focus on identifying phenotypes associated with a lack of KISS1 or GnRH signaling in skeletal muscle; KISS1/GnRH is a potential therapeutic target to attenuate heightened inflammation in disease states.

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CHAPTER 3. DISCUSSION

3.1 Summary of the relationship between KISS1/GnRH signaling and inflammation in skeletal muscle

Chronic muscle inflammation contributes to skeletal muscle atrophy and impaired regenerative capacity (1, 2). Skeletal muscle inflammatory susceptibility, or the ability to manage and respond to inflammation, has been identified as a predictor of failed skeletal muscle regeneration and regrowth following surgery (3, 4). This inflammatory susceptibility is associated with human aging and likely contributes to the adverse structural, metabolic, and functional tissue remodeling that occurs in aged adults (3). As the etiology for heightened inflammation in skeletal muscle is unclear, particularly in healthy young adults, research that elucidates the mechanisms by which inflammation impairs skeletal muscle regeneration is necessary for identifying potential therapeutic targets.

Previous research from our laboratory identified that kisspeptin-1 (KISS1) was the most highly differentially expressed gene (DEgene) between cells from donors with (MuIS+) and without (MuIS-) inflammatory susceptibility (downregulated 21.4-fold [MuIS+/MuIS-]) and was a node in the top network of DEgenes following pathway analysis (5). In addition to stimulating the release of gonadotropin-releasing hormone (GnRH) in the hypothalamus, KISS1 has been linked to inflammation in various cell types (6). KISS1 inhibits the tumor necrosis factor alpha (TNFα)-mediated activation of the proinflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway in breast cancer cells (7), reduces NF-κB binding to the promoter in fibrosarcoma cells (8), and inhibits cancer metastasis in multiple other cancers (6, 9, 10, 11, 12). Interestingly, GnRH signaling emerged as a top canonical pathway common in both the MuIS- myocytes and in individuals who exhibited hypertrophic responses to resistance

exercise training (13, 14). In the present study, we aimed to investigate the potential relationship between KISS1/GnRH signaling and inflammation in skeletal muscle.

We confirmed the presence of the GnRH receptor (GnRHr) in skeletal muscle with a gene detection assay using skeletal muscle obtained from ROSA26/GRIC mice. This assay also revealed potential sex differences that require further exploration. We used differentiated human primary muscle stem cells (i.e., myocytes) and differentiated immortalized murine myoblast cells (C2C12 myocytes) treated with Buserelin, a GnRH analog, to investigate canonical downstream GnRH/GnRHr signaling through the mitogen-activated protein kinase (MAPK) pathways: extracellular-regulated kinase (ERK), Jun N-terminal kinase (JNK), and the downstream transcription factor cAMP response element-binding protein (CREB). The MAPK signaling pathways regulate cellular processes including cell proliferation, differentiation, and survival or apoptosis (15, 16). The addition of Antide, a competitive inhibitor that binds GnRHr, diminished CREB activation at 60 min compared to Buserelin alone, suggesting GnRH/GnRHr signaling, through CREB, may be functional in skeletal muscle. Results from searching NCBI GEO datasets revealed that KISS1/GnRH signaling may be downregulated in various inflammatory myopathies. However, this concept was not supported in myocyte culture when we treated myocytes with Buserelin and TNFα, a pro-inflammatory cytokine, prior to measuring inflammatory cytokine mRNA levels.

Our methods may have been insufficient to detect the transient length of time that Buserelin would activate MAPK pathways or influence the TNFα-mediated increase in IL-6 mRNA levels. Due to time constraints and limitations of commercial antibodies currently available, KISS1, GnRH, or GnRHr protein levels could not be examined in this study. It is possible that the association between KISS1 and inflammation in skeletal muscle is independent of GnRH.

3.2 Unexplored pathways potentially associated with KISS1/GnRH in skeletal muscle

There are additional mechanisms by which KISS1 and/or GnRH may regulate inflammation in skeletal muscle that were not explored in the present study. For example, matrix metalloproteases (MMPs) are a class of enzymes involved in the regulation of extracellular matrix degradation in many tissues, and regulate cell migration, differentiation, and regeneration in skeletal muscle (17). The activity of MMP-9, expressed in polymorphonuclear leukocytes and macrophages, is upregulated for the first three days following skeletal muscle injury and aids in satellite cell activation. Bani et al. (2008) found that inflammation and necrosis of the skeletal muscle tissue was associated with an increase in the activity of MMP-9 and TNFα, and that increased MMP-2 activity may reflect the extent of disease progression in dystrophic muscle (18). Both MMP-2 and MMP-9 participate in post-translational modification of KISS1. KISS1 binding to KISS1r has been shown to dephosphorylate NF-κB in fibrosarcoma cells, resulting in dissociation from the MMP-9 promoter via a MAPK-independent mechanism (8). In addition, MMP-9 expression was reduced in colorectal cancer cells with the addition of KISS1 and an ERK1/2 inhibitor (10). It is plausible KISS1 exerts a regulatory effect on NF-κB through MMP-9 in skeletal muscle, independent of MAPK signaling.

Calcium signaling emerged as the most significant and highly activated canonical pathway in MuIS- myocytes compared to MuIS+ myocytes and several components of the calcium signaling pathway were upregulated in MuIS- cells. Additionally, two top pathways shared by MuIS- and extreme responders to resistance training included GnRH signaling and calcium signaling. Calcium, which exerts allosteric effects on a wide array of intracellular proteins and functions as a signal transducer in most body tissues, is necessary for myogenesis. Satellite cell recruitment, activation, and proliferation triggered by mechanical stimuli is ablated

with inhibition of L-type calcium channels (19). Transcription factors, including CREB and other MAPK-stimulated factors, transmit the calcium signal in skeletal muscle. Stimulus-driven calcium-dependent phosphorylation of ERK and CREB leads to an upregulation of the genes c-FOS, c-JUN, and EGR1. Calcium release mediated by type 1 inositol 1,4,5-trisphosphate receptors is essential for human myoblast differentiation, but not for muscle regeneration (19). The mechanisms regulating calcium signaling during satellite cell activation and muscle regeneration have yet to be fully elucidated. Dysregulated calcium signaling has also been linked to cachexia and other inflammation-centered myopathies such as myositis (20).

In the pituitary gonadotrope, GnRH is a calcium signaling modulator. GnRH action requires calcium to activate ERK, which subsequently facilitates L-type calcium influx (21, 22). GnRH-mediated intracellular mobilization of calcium activates calcium-calmodulin-dependent protein kinases (CaMK) and the calcium-calmodulin-dependent phosphatase calcineurin. The expression of calcineurin is required for skeletal muscle differentiation and is upregulated along with voltage-gated calcium channels in MuIS-/MuIS+ myocytes (23). In systems such as the pituitary and clonal gonadotrope cells, GnRHr couples to Gαs and stimulates cAMP. Activation of calcium/calmodulin-sensitive adenylyl cyclase isoforms independent of Gαs has been proposed as the mechanism of GnRHr-induced cAMP elevation (21, 24, 25). It is possible that GnRH acts in a calcium-dependent manner to activate ERK and regulate proinflammatory pathways in skeletal muscle.

Actin cytoskeleton development is a critical process in skeletal muscle regeneration, essential for normal cell division during differentiation, myoblast fusion, muscle elongation and attachment, muscle maturation, and sarcomere assembly (26). GnRH signaling and actin cytoskeleton signaling emerged as top canonical pathways shared by MuIS- myocytes and extreme responders to resistance training. GnRH signaling has been shown to directly induce

actin cytoskeleton remodeling and cell migration in prostate cancer cell lines (27). There is also evidence that KISS1 itself acts downstream of cell-matrix adhesion involving cytoskeletal reorganization, as well as in positive regulation of cytosolic calcium ion concentration (9, 28). Cytoskeleton remodeling has been proposed as a mechanism of KISS1/KISS1r anti-metastatic action in breast cancer cells (9). We have shown for the first time that KISS1/KISS1r signaling is downregulated in several myopathies, and the majority of skeletal muscle myopathies are directly linked to a lack of function in cytoskeletal components (26). It is plausible that KISS1 and/or GnRH contribute to myogenesis via actin cytoskeleton development and remodeling.

The p38 MAPK pathway is also a potential mediator of KISS1/GnRH signaling in skeletal muscle. p38 regulates myogenesis during satellite cell activation and asymmetric division, proliferation, and differentiation (18, 29, 30). Bernet et al. (2014) showed that p38 plays a crucial role in self-renewal of satellite cells; satellite cells from aged mice fail to regain self-renewal capacity following transplantation into a young microenvironment, likely due to dysregulated p38 MAPK signaling (31). It is possible that KISS1 and/or GnRH acts through p38 MAPK to maintain homeostatic MAPK signaling during inflammation in skeletal muscle.

In addition to KISS1, the transcription factor early growth response 1 (EGR1) was identified as another top node in MuIS- compared to MuIS+ myocytes. EGR1 regulates cell differentiation, growth, inhibition of growth, and apoptosis (32, 33). KISS1 induces transcription of EGR1 in gonadotropes *in vitro* (34) and EGR1 has antiproliferative effects in pancreatic cancer cells alongside the anti-metastasis effects of KISS1 (35). Zhang et al. (2017) found that EGR1 promotes differentiation of bovine skeletal muscle-derived satellite cells by upregulating gene expression of Myogenin, a transcription factor required for myogenesis (36). In the early inflammatory stage of the skeletal muscle repair process, EGR1 is activated by IL-1β and is

associated with IL-6 following crush injury *in vivo* (37, 38, 39). EGR1 is a potential transcription factor that KISS1 could act through to regulate inflammatory signaling during myogenesis.

Apart from KISS1, several hormones central to the mammalian reproductive system have been shown to also regulate skeletal muscle homeostasis and regeneration under basal and inflammatory conditions. Oxytocin, best known for its role in lactation, parturition and behavior, is required for skeletal muscle homeostasis and successful regeneration (40). A genetic knock-out of oxytocin in mice resulted in premature muscle atrophy and impaired muscle function (40). Estrogen, the primary female sex hormone, is also known to stimulate muscle repair and regeneration, including SC activation and proliferation. Prolonged estrogen deficiency in female mice leads to a shift towards glycolytic myofiber types, impaired SC function, diminished regenerative capacity, and muscle atrophy (41). Estrogen alleviates the loss of skeletal muscle mass associated with sepsis and blunts sepsis-induced hypothalamic inflammation and dysregulation of hypothalamic neuropeptides (42). In post-menopausal women, estrogen lowered training-induced muscle damage (43). Our previous research showed that β-estradiol signaling was predicted to be activated as an upstream regulator in MuIS-, suggesting that β-estradiol is elevated in the skeletal muscle of MuIS- individuals prior to the observed increase in KISS1 gene expression. In the hypothalamus, estrogen exerts both positive and negative feedback regulation on GnRH release through KISS1 neurons (44). It is feasible that estrogen regulates KISS1 expression in skeletal muscle.

3.3 Future directions

Although our research suggests a potential link between KISS1/GnRH signaling and inflammatory myopathies, we did not identify the role(s) of these hormones in skeletal muscle. Future research should focus on identifying phenotypes associated with a lack of KISS1 in

skeletal muscle. A KISS1 knockdown *in vitro* model using small interfering RNA (siRNA) or short hairpin RNA (shRNA) or a muscle-specific KISS1 knockout *in vivo* model could contribute to this line of questioning. Due to the lack of sufficient commercial antibodies targeting KISS1, KISS1r, GnRH, and GnRHr, the levels of these proteins could be determined in cell lysate from an *in vitro* model or tissue homogenate from an *in vivo* model using liquid chromatography–mass spectrometry (LC-MS). To determine the nature of the relationship between KISS1 or GnRH signaling and inflammation in skeletal muscle, the expression of proinflammatory genes in the tissue homogenate from an *in vivo* KISS1 knockout model could be assessed using RNA sequencing. In further experiments to determine mechanistic pathway by which KISS1/GnRH signals in skeletal muscle, ELISA would be a useful method to measure transient ERK phosphorylation.

As the results of searching the NCBI GEO datasets revealed that KISS1 and/or GnRH may be associated with myopathies including Duchenne muscular dystrophy, juvenile dermomyositis, tibial muscular dystrophy, facioscapulohumeral dystrophy, myotonic dystrophy type 2, and amyotrophic lateral sclerosis, future research should investigate the effects of a KISS1 knockout or overexpression *in vivo* model of an inflammatory myopathy. Our results suggest a striking downregulation of KISS1 in skeletal muscle from individuals with JDM compared to healthy controls. A transgenic mouse model of myositis, such as the model developed by Li et al. (2009), may be the most appropriate model to study downstream effects of genetic disruption of KISS1 (45).

In conclusion, our results confirmed that GnRHr protein is present and functional in skeletal muscle and that KISS1/GnRH have a relationship with inflammation that is impacted with muscle inflammatory susceptibility and myopathies. In addition to MAPK signaling, KISS1/GnRH may impact inflammation in skeletal muscle through the regulation of MMP-9,

calcium signaling, actin cytoskeleton remodeling, p38 MAPK, and/or EGR1. It is possible that estrogen regulates KISS1 expression in skeletal muscle. Future research should focus on identifying phenotypes associated with a genetic lack of KISS1 signaling in skeletal muscle and investigate the role of KISS1 in an *in vivo* model of inflammatory myopathy.

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